Universidade do Minho
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Doutoramento em Engenharia Química e Biológica

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Título da tese

Optimization of dark fermentation processes for biohydrogen production from sugars

Optimização da produção de bio-hidrogénio por fermentação anaeróbia de açúcares

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Summary

Hydrogen is recognized as a potential future energy carrier for the replacement of fossil fuels. Mixed culture dark fermentation is considered one of the most promising, sustainable and environmentally friendly process for byohydrogen production, since waste and wastewaters can be used as feedstocks. This thesis reports batch and continuous experiments performed at mesophilic and extreme thermophilic temperatures to optimize biohydrogen production by dark fermentation of sugars-rich wastewaters. Arabinose (pentose) and glucose (hexose) were used as model substrates.

In a first set of batch experiments, the response of suspended and granular biomass to increasing concentrations of arabinose (10 to 100 g L^{-1}) or different initial pH (between 4.5 and 8.0) was studied. Higher hydrogen rates and yields were observed for arabinose concentrations between 10 and 40 g L^{-1} and for higher pH values. Arabinose concentrations from 40 to 100 g L^{-1} inhibited hydrogen production. Granular biomass was less affected by increasing initial arabinose concentrations and can be used for larger pH ranges without losing its hydrogen production potential and arabinose uptake capacity, when compared with suspended sludges. Moreover, acclimated granular inocula showed the highest hydrogen production potential and percentage of arabinose consumption, suggesting the importance of biomass acclimatization for hydrogen production.

In the first continuous experiment, heat and chemical treatment (with 2-bromoethanesulfonate (BES) and with BES+Chloroform) was applied to suppress hydrogen-consuming microorganisms in granular sludge used to inoculate three mesophilic EGSB reactors. Pretreatment and subsequent pulse with BES was the best strategy, since it was effective in extinguishing the methanogenic hydrogenotrophic activity and did not extensively affect macro- and microstructure of the granules. A hydrogen production rate of 700 ± 200 $mLH_2L^{-1}d^{-1}$ was achieved in this reactor. Bacteria branching within *Clostridiaceae* and *Ruminococcaceae* were present in this sludge.

A second continuous experiment aimed at studying the effect of bioaugmenting granular sludge with an enrichment culture on the hydrogen production. Two EGSB reactors were individually inoculated with heat treated methanogenic granules (HTG) and HTG amended with an enrichment culture with high hydrogen production capacity (engineered heat treated methanogenic granules - EHTG), respectively. The bioreactors were operated at 70°C. A significant improvement of hydrogen production and process stability was obtained in the EHTG system, comparatively to the HTG system. The presence of active hydrogen-producers in the EHTG system during the reactor start-up induced the development of an efficient H_2-producing bacterial community, characterized by the presence of *Clostridium* sp*; Klebsiella* sp*; Thermoanaerobacterium thermosacharolyticum; Bacillus coagulans.*

In the final experiment reported in this thesis, the conversion of arabinose and glucose to hydrogen, by extreme thermophilic anaerobic mixed cultures was studied in continuous (70°C, pH 5.5) and batch (70°C, pH 5.5 and pH 7) assays. No significant differences on the reactors' performance were observed for low feed sugar-concentrations. However, for higher sugar loads, the reactor fed with arabinose showed better overall performance. The lower hydrogen production observed in the reactor fed with glucose was associated to a higher lactate production. The effect of hydrogen partial pressure and pH was studied in batch assays. An increase of hydrogen production from both substrates was observed when hydrogen partial pressure was kept low. Sugars uptake was enhanced and hydrogen production stimulated when pH 7 was used.

Sumário

O hidrogénio é reconhecido como um potencial vector energético para substituição dos combustíveis fósseis. A produção biológica deste combustível por fermentação anaeróbia usando culturas mistas é um dos processos mais promissores, ambiental e economicamente sustentáveis, uma vez que podem ser utilizados como matéria-prima para a produção de biohidrogénio resíduos e efluentes orgânicos. Nesta tese são descritos estudos efectuados em reactor fechado e em reactor contínuo a temperaturas mesofílicas e termofílicas com o objectivo de optimizar a produção de biohidrogénio a partir de efluentes sintéticos ricos em açúcares. Como substratos modelo, foram usados dois açúcares, arabinose (pentose) e glucose (hexose).

Numa primeira serie de experiências efectuadas em reactor fechado, foi estudada a resposta, em termos de produção de hidrogénio, de biomassa suspensa e granular sujeita a concentrações crescentes de arabinose (10 a 100 g L^{-1}) e a diferentes valores iniciais de pH (entre 4.5 e 8.0). Os maiores rendimentos e taxas de produção de hidrogénio foram obtidos para concentrações de arabinose entre 10 e 40 g L^{-1} e pHs mais elevados (entre 6 e 8). Concentrações entre 40 a 100 g L^{-1} inibiram a produção de hidrogénio. Verificou-se que a biomassa granular foi menos afectada pelas concentrações crescentes de arabinose, podendo ser usada num maior intervalo de pHs, sem perder contudo, a capacidade de produção de hidrogénio e utilização de arabinose. Adicionalmente observou-se que a biomassa granular aclimatizada apresentava um maior potencial para produção de hidrogénio e consumo de arabinose, mostrando deste modo, a importância da aclimatação da biomassa para a produção de biohidrogénio.

No primeiro estudo efectuado em reactor contínuo, foram utilizados três tratamentos, um térmico e dois químicos (com 2-bromoetanosulfonato (BES) e com BES+clorofórmio) para inibir a actividade dos microrganismos consumidores de hidrogénio presentes na biomassa granular. Posteriormente, as biomassas tratadas, foram utilizadas como inóculo de três reactores EGSB. O pré-tratamento e subsequente pulso com BES revelou ser a melhor estratégia testada, uma vez que foi efectiva na eliminação da actividade hidrogenotrófica metanogénica, não tendo afectado severamente a macro e a micro estrutura dos grânulos. O reactor onde se utilizou esta estratégia alcançou uma taxa de produção de 700 ± 200 $mLH_2L^{-1}d^{-1}$. Verificou-se que a comunidade bacteriana dominante neste reactor pertencia às famílias *Clostridiaceae* e *Ruminococcaceae*.

No segundo estudo efectuado em contínuo, foi avaliado o efeito da bioaumentação de uma biomassa granular com uma cultura enriquecida em micorganismos produtores de hidrogénio. Um reactor EGSB foi inoculado com grânulos metanogénicos tratados termicamente (HTG) e o outro reactor com HTG juntamente com uma cultura enriquecida que apresentava grande capacidade de produção de hidrogénio (EHTG). Os dois reactores foram operados a 70°C. O sistema EHTG mostrou um aumento significativo na produção de hidrogénio e na estabilidade do processo, comparativamente ao sistema HTG. A presença de microrganismos activos, produtores de hidrogénio no início da operação do sistema EHTG induziu o posterior desenvolvimento de uma comunidade bacteriana eficiente na produção de hidrogénio. Esta comunidade caracterizou-se pela presença de *Clostridium* sp., *klebsiella* sp., *Thermoanaerobacterium thermosacharolyticum* e *Bacillus coagulans*.

Na última experiencia descrita nesta tese, foi estudada a conversão em reactor contínuo (70°C, pH 5.5) e em reactor fechado (70°C, pH 5.5 and pH 7) da arabinose e glucose a hidrogénio, usando culturas mistas em condições termofílicas extremas. Não foram observadas diferenças na eficiência dos reactores para concentrações baixas de

substrato. No entanto, para concentrações mais elevadas de substrato, o reactor alimentado com arabinose obteve melhor desempenho. A produção de hidrogénio mais baixa, observada no reactor alimentado com glucose, deveu-se ao aumento significativo de lactato. O efeito da pressão parcial de hidrogénio e do pH na produção de hidrogénio a partir destes dois açúcares foi estudado em reactor fechado, tendo-se observado um aumento da produção de hidrogénio, a partir de ambos os substratos, quando a pressão parcial de hidrogénio foi mantida baixa. A produção de hidrogénio e a utilização de ambos os açúcares foi estimulada com utilização do pH 7.

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List of Symbols and Abbreviations

AFBR - Anaerobic fluidized bed reactor

ASBR - Anaerobic sequencing batch reactor

ADP - Adenosine diphosphate

ATP - Adenosine triphosphate

BEAMR - Bioelectrochemically assisted microbial reactor

BES - 2-Bromoethanesulfonate

CIGSB - Carrier-induced granular sludge bed

C/N - Carbon-to-nitrogen ratio

C/P – Carbon to phosphate ratio

CoA - Coenzyme A

COD - Chemical oxygen demand

CSTR - Continuously stirred tank reactor

D - Dilution rate

DNA – Desoxyribonucleic acid

ΔG - Gibbs free energy change

EGSB - Expanded granular sludge bed

EU - European Union

F/M – Food to microorganisms

FBBAC - Fixed bed bioreactor with activated carbon

FBR - Fluidized bed reactor

$Fd_{(ox)}/Fd_{(red)}$ - Oxidized/reduced forms of ferredoxin

GC - Gas chromatography

GHG - Greenhouse gas

HRT - Hydraulic retention time

HPLC - High-performance liquid chromatography

H_2ase - Hydrogenase

IPCC - Intergovernmental Panel on Climate Change

MBR - Membrane bioreactor

MEC - Microbial electrolysis cells

MFC - Microbial fuel cells

NAD^+/NADH - Oxidized/reduced forms of nicotinamide adenine dinucleotide

NRB - Nitrate-reducing bacteria

OD – Optical density

OFMSW - Organic fraction of municipal solid waste

OLR - Organic loading rate

PCA - Principal components analysis

PCR – Polymerase chain reaction

PHAs - Polyhydroxyalkanoates

PH_2 - Hydrogen partial pressure

PLS - Partial least squares regression

rRNA - Ribosomal ribonucleic acid

rpm - Revolutions per minute

SEM -Scanning electron microscopy

SFP – Soluble fermentation products

SRB - Sulphate-reducing bacteria

SRT – Solids retention time

STP - Standard temperature and pressure

TBR - Trickling biofilter reactors

TVS – Total volatile solids

UASB - Upflow anaerobic sludge blanket

VFA - Volatile fatty acids

v - Volume

VS - Volatile solids

VSS - Volatile suspended solids

w – Weight

WW - Wastewater

WWTP - Wastewater treatment plant

Context, aim and thesis outline

Chapter 1

1.1 Perspectives and motivation

During the first centuries of mankind existence, the combustion of wood was enough to fulfill the energy requirements, which were limited to cooking, warming and protection. However, as the world population started to grow, more efficient energy production processes were needed, turning mankind to the use of coal and later of oil. Today, the overexploitation of fossil fuels has brought mankind to a dead end, from both an environmental and energy resource perspectives. Fossil fuel reserves are not endless. Consequently, their massive use, in order to cope with the continuous increase in energy demands, brought the humanity to an imminent world energy crisis (Barnham et al., 2006). Moreover, excessive accumulation of greenhouse gases, specially carbon dioxide, in the atmosphere has been occurring (Conte et al., 2001). The intergovernmental Panel on Climate Change (IPCC) reported that the emission of global greenhouse gas (GHG) increased 70% between 1970 and 2004 as the result of human activities. Therefore, an urgent need for identifying and exploiting alternative energy sources has emerged, with a special focus on renewable and environmental friendly sources.

As a sustainable energy supply with minimal or zero use of hydrocarbons, hydrogen is a promising alternative to fossil fuels. Although not readily available in its molecular form, hydrogen is quite abundant in nature, and has the highest energy yield (122kJ g^{-1}) comparatively to other fuels (van Groenestijn et al., 2002). Hydrogen has multiple potential applications as energy carrier. It can be used as a vehicle fuel, for stationary production of electricity, heat and as a fuel in portable electronics. Annually, about 50 million tons of hydrogen are produced globally (National Hydrogen Association, 2010), and are mainly used as a feedstock in the industry, with very limited use as fuel or energy carrier. The major industrial applications of hydrogen, include manufacturing ammonia (about 60%) and oil refining (cracking and hydrogenation of hydrocarbons) (Ramachandran and Menon, 1998). The key drivers and barriers of hydrogen economy are related to the immaturity of the technology, the lack of commercially competitive technologies and infrastructures, and economical

risks.

Hydrogen can be produced from renewable sources via a number of processes and it is a clean and environmentally friendly fuel that produces water instead of greenhouse gases when combusted. Those characteristics make hydrogen an appealing candidate for the future energy economy.

1.2 Hydrogen production

Hydrogen can be produced from a variety of feedstocks. These include fossil resources, such as natural gas and coal, as well as renewable resources, such as biomass and water, with input from renewable energy sources (e.g. sunlight, wind, wave or hydro-power) (Figure 1.1). A variety of process technologies can be used, including chemical, biological, electrolytic, photolytic and thermo-chemical. Each technology is in a different stage of development, and each offers unique opportunities, benefits and challenges. Local availability of feedstock, the maturity of the technology, market applications and demand, policy issues and costs will all influence the choice and timing of the various options for hydrogen production. Globally, 40% of hydrogen is produced from natural gas, 30% from crude oil, 18% from coal, and 4% from water electrolysis (Das, 2009).

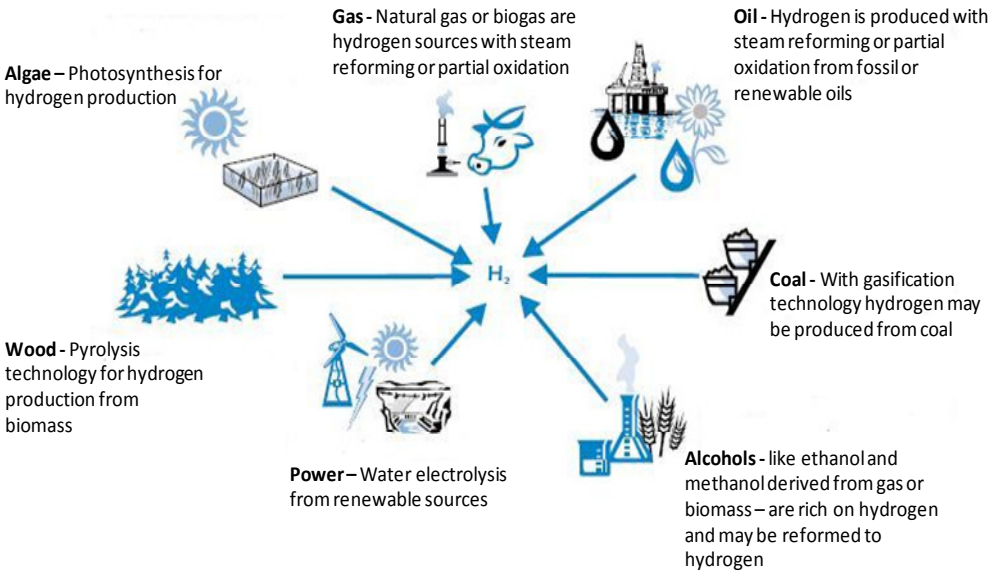

Figure 1.1. - Some feedstock and process alternatives for hydrogen production (adapted from OECD/IEA, 2006)

Biological hydrogen production (biohydrogen) has the potential to considerably reduce costs and environmental impact as it can be produced with sunlight and minimal nutrients or from organic wastes. Hydrogen producing microorganisms can be rapidly grown in bioreactors with relatively small energy and environmental footprints, making biohydrogen production a renewable and low impact technology. It may also be considered as a CO_2 offset, because it utilizes carbon sources that are already present in the environment (Brentner et al., 2010).

1.3 Biological hydrogen production

Biological processes for hydrogen generation include biophotolysis, indirect biophotolysis, photofermentation and dark fermentation of organic matter (Brentner et al., 2010). Table 1.1 provides a comparison between these mechanisms.

Recently, microbial electrolysis cells (MEC), also called bioelectrochemically assisted microbial reactors (BEAMR), have been also used to produce hydrogen from biodegradable material (Cheng and Logan, 2007). In this process, exoelectrogenic bacteria transfer electrons to the anode and protons into the solution while oxidizing organic matter. Electrons flow to the cathode through an external wire where they are combined with protons to form hydrogen gas. A small electrical input is added, in addition to that supplied by the bacteria to overcome the endothermic barrier of hydrogen formation.

Additionally, hydrogen may be produced by certain bacteria from *Rhodospirillacae* family. These microorganisms are able to grow in the dark by feeding only with CO (Levin et al., 2004). The oxidation of CO to CO_2 was determined to follow the water-gas-shift reaction ($CO+H_2O \longrightarrow CO_2+H_2$), using enzymes.

Significant advances have been made over the past two decades in biohydrogen production. Different European Union projects have been developed aiming the optimization of biohydrogen production, such as, BIOHYDROGEN and

HYVOLUTION. In the BIOHYDROGEN project, the main objective was to produce hydrogen from energy crops and wastes, employing hyperthermophilic and photoheterotrophic microorganisms to supply the fuel cell industry with clean hydrogen gas. (http://www.biohydrogen.nl/biohydrogen). The HYVOLUTION assessed the potential of sugar beet to produce biohydrogen in the EU with particular focus on two member states – the Netherlands and Greece. It was achieved 204 millimoles of hydrogen per liter of sugar beet juice using bacterium *Caldicellulosiruptor saccharolyticus* DSM8903. (http://www.biohydrogen.nl /hyvolution/31827/9/0/20).

Several goals have been achieved in biohydrogen production, like the identification of hydrogen-producing microorganisms, engineering of microorganisms to improve H_2 production efficiency and systems' optimization to maximize growth and hydrogen production potential. However, biohydrogen production is still not competitive at full scale, comparing to other technologies that are currently in use for hydrogen production. Current biohydrogen technology is not ready for industrial scale production and a decentralized strategy may be more appropriate to reduce costs associated with transport and storage of hydrogen. The next phase of biohydrogen research needs to include pilot scale demonstration projects to explore the opportunities for industrial scale production.

1.4 Dark fermentation biohydrogen production

In dark fermentation, carbohydrate-rich substrates are converted by anaerobic microorganisms into organic acids and alcohols, releasing hydrogen and CO_2 (Figure 1.2). A variety of microorganisms can be involved in this process, either as pure or mixed cultures.

Reactors are typically seeded with a mixture of undefined microorganisms and the strains for biohydrogen dark fermentation are selected by the feedstock used and the reactor conditions applied (residence time, temperature, pH, etc).

Table 1.1. - Comparison of main biological processes for hydrogen production (adapted from Brentner et al., 2010; Hallenbeck and Ghosh, 2009)

Process	Organisms	Reaction	Advantages	Disadvantages	Maximum reported rate (mL H_2 L^{-1} h^{-1})	Reference
Direct biophotolysis	Green algae, cyanobacteria	$2H_2O \xrightarrow{\text{Light energy}} 2H_2 + O_2$	Abundant substrate (water); simple products (H_2 and CO_2)	Low light conversion efficiencies; oxygen-sensitive hydrogenase; expensive hydrogen impermeable photobioreactors required	11.7	(Laurinavichene et al., 2006)
Indirect biophotolysis	Cyanobacteria	$12H_2O + 6CO_2 \xrightarrow{\text{Light energy}} C_6H_{12}O_6 + 6CO_2$ $C_6H_{12}O_6 + H_2O \xrightarrow{\text{Light energy}} 12H_2 + 6CO_2$	Abundant substrate (water)	Bioreactor design challenges to maximize utilization of sunlight	13.8	(Lindblad et al., 2002)
Photofermentation	Purple nonsulfur bacteria	$C_xH_yO_z + (2x - z)H_2O \xrightarrow{\text{Light energy}} (y/2 + 2x - 2)H_2 + xCO_2$	Utilizes energy from sunlight to convert small organic acids or waste organic compounds to H_2 and CO_2 with no byproducts	Low light conversion efficiencies; expensive hydrogen impermeable photobioreactors required; nutrients required	82.5	(Kim et al., 2006)
Dark fermentation	Anaerobic bacteria	$C_6H_{12}O_6 + H_2O \longrightarrow 2CH_3COOH + 4H_2 + CO_2$	No direct solar input needed; variety of waste streams/energy crops can be used; simple reactor technology	Low H_2 yields; incomplete substrate utilization	9300	(Lee et al., 2006)
Water gas shift reaction	Bacteria from *Rhodospirillacae* family	$CO+H_2O \longrightarrow CO_2+H_2$	Convert directly CO to CO_2 and H_2	Low H_2 yields; require a CO source and darkness	2.46	(Levin et al., 2004)
Microbial electrolysis cells (MEC)	Anaerobic bacteria	$2H^+ + 2e^- \longrightarrow H_2$	Convert directly biodegradable material into hydrogen	Low H_2 yields	0.059	(Cheng and Logan, 2007)

The hydrogen consuming organisms (such as methanogenic archaea, homoacetogens, nitrate and sulphate reducers) must be suppressed and the microbial consortium that develops in a reactor will affect the distribution of the byproducts, including the make-up of the gas. Hydrogen-producing sludges are often dominated by *Clostridium,* enteric and *Bacillus* species (Fang et al., 2002a; Fang et al., 2002b; Wang et al., 2003). Mixed communities of microorganisms are generally more stable and adaptable to changes in environment and feedstock than pure cultures, making them better suited for continuous operations and waste stream applications.

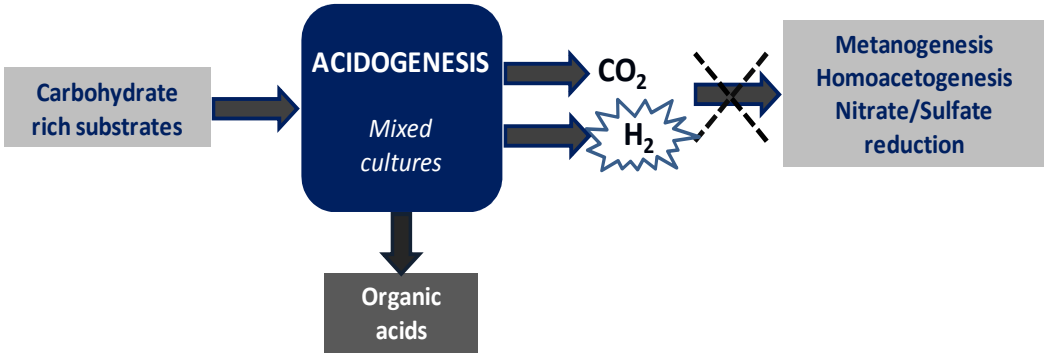

Figure 1.2. – Schematic representation of dark fermentation biohydrogen production.

The use of dark fermentation presents several advantages over the other biohydrogen production processes such as higher hydrogen generation rates, continuous hydrogen production at a sustained rate since it does not depend on light energy, and generation of commercially valuable metabolites (such as organic acids) (Table 1.1).

The most important advantage of the dark fermentation process is that substrate can be supplied by several carbohydrate and/or starch-rich wastes/wastewaters, as well as cellulose-rich biomass, combining the dual function of partial treatment and energy production (Das and Veziroglu, 2001; Levin et al., 2004; Ramachandran and Menon, 1998). Different wastes and biomass have been tested as potential feedstock, leading in many cases to very promising results, as described in sub-section 2.6.4.

The major limitations of dark fermentation remain the relatively low yields and rates of hydrogen generation comparing to non-biological hydrogen production methods. To overcome this drawback, fermentative hydrogen producing systems can potentially be combined with a second stage process, in which the emerging byproducts can be used for extra energy and/or materials recovery, either via hydrogen, biogas or other valuable products in a biorefinery framework.

Future progress leading to more efficient fermentative biohydrogen production will depend on research efforts to identify the most suitable feedstocks, isolate and/or develop efficient hydrogen-producing strains through genetic engineering, and optimize reactor configurations and operating strategies.

1.5 Research aim

The aim of the research presented in this thesis was to optimize biohydrogen production in dark fermentation processes. C5 and C6 sugars were used as substrate, and several strategies for selection of H_2-producing microorganisms, bioreactors' start-up strategies and operating conditions were tested. Classical reactors' performance parameters, namely biohydrogen production, yield and fermentation products were complemented with the study of morphological properties of granular sludge and microbial communities.

1.6 Outline of this thesis

The focus of this thesis, and its relevance in the framework of biohydrogen production was introduced in the present chapter. A general overview on the current knowledge about biohydrogen dark fermentation is further presented in **Chapter 2**. A special focus is given to fundamental knowledge of anaerobic fermentative biohydrogen production, microorganisms involved, bioprocess parameters, suitable feedstocks, type of bioreactors and second stage processes.

In **Chapter 3** the response of suspended and granular sludge to increasing concentrations of arabinose, a pentose sugar present in hemicellulosic materials,

is described. Additionally, the effect of a wide range of initial pH values on the biohydrogen production from arabinose by suspended and granular sludge was evaluated, as presented in **Chapter 4**. The influence of the type of inoculum and the relationship between different parameters were studied by principal component analysis and partial least square analysis.

In **Chapter 5**, different strategies to suppress hydrogen-consuming microorganisms from already formed mesophilic methanogenic granules are presented. The effect of those strategies on the macro and micro-scale structural morphology of granular sludge was evaluated, resulting in the establishment of a comprehensive link between granules morphology and the microbial community structure.

A comparison of two different reactor start-up strategies at extreme thermophilic conditions is presented in **Chapter 6.** One of the strategies consisted on the use of an "engineered inocula", where known hydrogen producers were co-inoculated with heat treated methanogenic granules; the other used heat treated methanogenic granules as inoculum. The two strategies were compared in terms of hydrogen production rate, process stability and bacterial community structure.

In **Chapter 7** the conversion of arabinose and glucose to hydrogen, by extreme thermophilic anaerobic mixed cultures is described. Two bioreactors, continuously fed with arabinose and glucose were compared in terms of hydrogen production rate, process stability and bacterial community structure. Additional batch experiments were performed to study the effect of hydrogen partial pressure and pH on the hydrogen production from both sugars.

Chapter 8 contains a general discussion and concluding remarks as well as perspectives for further research in this topic.

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Fundamentals of Biohydrogen Dark Fermentation

Chapter 2

Abstract

Intensive research on hydrogen dark fermentation is underway and in the last years several novel approaches have been proposed and studied. Advances in bioreactor engineering, environmental parameters optimization and metabolic engineering have been carried out to improve hydrogen production yields and rates.

This literature review provides the state of the art on hydrogen dark-fermentation, focusing on fundamental knowledge of anaerobic hydrogen production, microbiology and biochemistry of the process, environmental parameters, suitable feedstocks, type of bioreactors and second stage processes.

2.1 Hydrogen in anaerobic environments

In dark fermentation, obligate and facultative anaerobic microorganisms break down complex organic matter into organic acids and alcohols, releasing H_2 and CO_2. For that, proteins, lipids and carbohydrates are firstly hydrolyzed to monomeric molecules (Figure 2.1), and after, fermentative bacteria produce organic acids, H_2 and CO_2 from the monomeric molecules. At that point, acetate and H_2 can be utilized and/or produced by several microbial groups. Acetate can be generated during the autotrophic acetogenesis via Wood-Ljungdahl pathway from CO_2 reduction and hydrogen, namely homoacetogenesis (Muller, 2003). Syntrophic bacteria generate acetate and hydrogen from short chain organic acids.

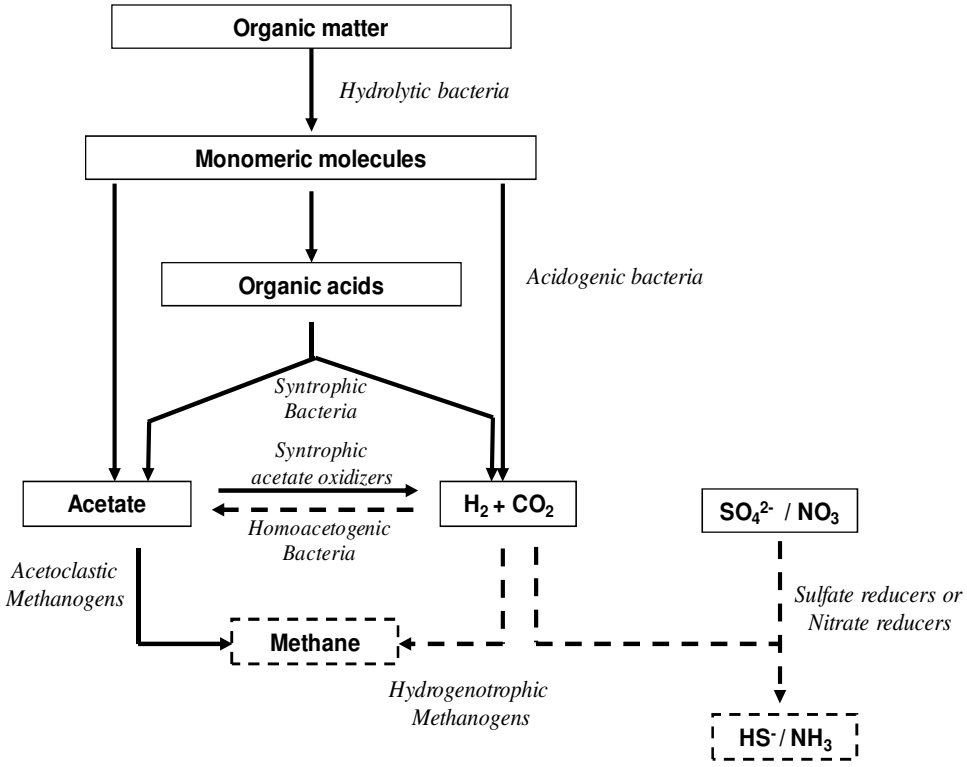

Figure 2.1. - Anaerobic degradation of organic matter (scheme adapted from Garcia et al., 2000)**.**

For the complete degradation of organic matter, the consumption of organic acids and hydrogen is essential, and is generally accomplished by acetoclastic/hydrogenotrophic methanogens producing CH_4 and CO_2 (Garcia et

al., 2000). In the presence of sulphate or nitrate, sulphate-reducing bacteria (SRB) or nitrate-reducing bacteria (NRB) are capable of using hydrogen as electron donor generating sulphides and ammonia, respectively. Thus, hydrogen is a key intermediate consumed mainly by methanogens, SRB, NRB and homoacetogens. The hydrogen consumption enables biochemical reactions carried out by syntrophic bacteria to become exergonic and syntrophs can produce additional hydrogen from organic acids (Thauer et al., 1977). In consequence, hydrogen concentration and the activity of hydrogen-utilizing microorganisms may regulate the fermentative pathways.

Table 2.1. – Hydrogen-producing and hydrogen-consuming reactions in anaerobic processes

Reaction	Equation	$\Delta G^{0\prime}$ (kJ reaction^{-1})	References
Complete oxidation of glucose	$C_6H_{12}O_6 + 12H_2O \longrightarrow 6HCO_3^- + 6H^+ + 12H_2$	+3.2	(Thauer et al., 1977)
Acetate production	$C_6H_{12}O_6 + 4H_2O \longrightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2$	-206.3	(Thauer et al., 1977)
Butyrate production	$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3CH_2CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2$	-254.8	(Thauer et al., 1977)
Ethanol production	$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3CH_2OH + 2HCO_3^- + 2H^+$	-235.0	(Ren and Gong, 2006)
Lactate production	$C_6H_{12}O_6 \longrightarrow 2CH_3CHOHCOO^- + 2H^+$	-198.1	(Kim et al., 2006c)
Butanol production	$C_6H_{12}O_6 + H_2O \longrightarrow CH_3CH_2CH_2OH + 2HCO_3^- + 2H^+$	-280.5	(Chin et al., 2003)
Propionate production	$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3CH_2COO^- + 2H_2O + 2H^+$	-359.0	(Hussy et al., 2003)
Valerate production	$C_6H_{12}O_6 + H_2 \longrightarrow CH_3CH_2CH_2CH_2COO^- + HCO_3^- + H_2O + 2H^+$	-330.9	(Ren and Gong, 2006)
Homoacetogenesis	$4H_2 + 2HCO_3^- + H^+ \longrightarrow CH_3COO^- + 4H_2O$	-104.6	(Thauer et al., 1977)
Acetogenesis	$C_6H_{12}O_6 \longrightarrow 3CH_3COO- + 3H^+$	-310.6	(Kim et al., 2006a)
Hydrogenotrophic methanogenesis	$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$	-131.0	(Schink, 1997)
Anaerobic oxidation of acetate (synthrophic)	$CH_3COO^- + H_2O \longrightarrow 2HCO_3^- + H^+ + 4H_2$	+104.6	(Stams, 1994; Thauer et al., 1977)
Anaerobic oxidation of butyrate (synthrophic)	$CH_3CH_2CH_2COO^- + 10H_2O \longrightarrow 4HCO_3^- + 3H^+ + 10H_2$	+257.3	(Stams, 1994; Thauer et al., 1977)
Sulphate reduction	$SO_4^{2-} + 4H_2 + H^+ \longrightarrow HS + 4H_2O$	-151.0	(Schink, 1997)

Due to rapid hydrogen consumption, its concentration is usually extremely low and microorganisms have to compete for it. Therefore, establishment of one type of hydrogen consumer depends mainly on the type of inoculum, microorganisms' affinity for the substrate, hydrogen concentration, carbon source and solubility of the electron acceptor.

Under the best growth conditions for all hydrogen consumers, the major capacity to utilize low hydrogen concentrations is related to a more energetically favorable biochemical reaction (Cordruwisch et al., 1988a). Microorganisms that use nitrate as electron acceptor are the most efficient in using hydrogen at very low concentrations. In spite of this, NRB and/or SRB can only compete with methanogens and homoacetogens for the available hydrogen when hydrogen is the limiting resource and sulphate and nitrate are in excess (Weijma et al., 2002).

In most environmental situations, the Gibbs free energy change of CO_2 reduction to CH_4 (per carbon) is higher than that of CO_2 reduction to acetate (Schink, 1997). It has been shown that the minimum H_2 partial pressures in pure cultures of methanogens are roughly one order of magnitude lower (3 to 10 Pa) than those achieved by homoacetogens (50 to 100 Pa) under similar conditions (Cordruwisch et al., 1988b; Stams, 1994). This probably explains why methanogenesis usually predominates over homoacetogenesis as the terminal electron sink reaction (Schink, 1997). Only at lower temperatures the thermodynamic advantage change in favor of homoacetogenesis, and also in slightly acidic environments, methanogens may not be as competitive as homoacetogens as a hydrogen sink (Schink, 1997; Stams, 1994). For this reason, hydrogenotrophic methanogens are the main hydrogen-consuming microorganisms in most anaerobic environments (Morvan et al., 1996; Sterling et al., 2001; Weijma et al., 2002).

2.2 Metabolic pathways for hydrogen production

Biohydrogen production via dark fermentation is generally achieved through the activity of strictly anaerobic or facultative bacteria, under anaerobic conditions

(see section 2.6). Different organic substances, such as carbohydrates, sugars, proteins and lipids can in principle be used as substrates, although the reaction of glucose biotransformation towards acetate is widely accepted as reference for the estimation of theoretical yields of fermentative hydrogen production. According to that reaction (1) the maximum theoretical yield of biohydrogen from glucose fermentation is 4 mol of H_2 per mol of glucose consumed.

$$C_6H_{12}O_6 + 4H_2O \longrightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \quad (1)$$

The maximum hydrogen theoretical yield of 4 mol of H_2 per mol of glucose consumed can be also achieved in two steps via fermentation of glucose towards acetate and formate according to the reactions (2) and (3):

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COO^- + 2HCOO^- + 4H^+ + 2H_2 \quad (2)$$

$$2HCOOH \longrightarrow 2CO_2 + 2H_2 \quad (3)$$

When butyrate is the fermentation product, the maximum hydrogen theoretical yield becomes 2 mol of H_2 per mol of glucose consumed, according to the reaction (4):

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3CH_2CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2 \quad (4)$$

The basic step in all the above reactions is the metabolism of glucose towards pyruvate as shown in reaction (5) and Figure 2.2.

$$C_6H_{12}O_6 + 2NAD^+ \longrightarrow 2CH_3COCOO^- + 4H^+ + 2NADH \quad (5)$$

Subsequently, 2 mol of hydrogen can theoretically be generated during the regeneration of the reduced form of the nicotinamide adenine dinucleotide (NADH) produced ($NADH + H^+ \longrightarrow NAD^+ + H_2$).

The metabolic pathways for the fermentation of carbohydrates to hydrogen, CO_2, organic acids, alcohols and solvents are well understood (Madigan, 2000). The two main fermentation pathways include Embden-Mayerhof (EM) and Entner-Doudoroff (ED) pathways.

Clostridia and enteric bacteria, the main groups of dark fermentative hydrogen-producers, use EM pathway in the oxidation of carbohydrates (Wiegel, 1980). During the Embden-Meyerhof pathway (glycolysis), 1 mol of hexose is

metabolized to 2 mol of pyruvate with the production of 2 moles of reduced NADH and 2 mol of adenosine triphosphate (ATP) (Figure 2.2). *Clostridia* and enteric bacteria can also utilize the pentose phosphate pathway for the conversion of 3 mol of pentose to 5 mol of ATP and 5 mol of NADH (Rogers, 1986). Pentose sugars are fermented to pentose 5-phosphate. Then, by means of the transketolase-transaldolase sequence, fructose 6-phosphate and glyceraldehyde 3-phosphate are produced and can enter to glycolityc pathway (Figure 2.2).

Glycolysis only occurs in the microbial cells if the cytoplasmic electron carrier nicotinamide adenine dinucleotide (NADH) is constantly reoxidized. However, fermentative bacteria face a problem of disposing electrons provided in the oxidation of electron donors because they lack the ability of utilizing terminal electron acceptors (Madigan, 2000). One way to regenerate NAD^+ by these organisms include the formation of H_2 and/or reduced products such as lactate, ethanol, butyrate, succinate and propionate (Stams, 1994). In addition, to serve in the regeneration of NAD^+, the formation of butyrate, succinate and propionate is coupled with the formation of ATP through substrate level phosphorylation. The formation of acetate is coupled with the formation of ATP, but not with the regeneration of NAD^+ (Figure 2.2).

Among enteric bacteria and *Clostridia*, two different enzymatic systems are used for the metabolism of pyruvate formed during glycolysis. Enteric bacteria use pyruvate formate lyase (PFL) (Knappe et al., 1974), while *Clostridia* use pyruvate ferredoxin oxidoreductase systems (Uyeda and Rabinowi, 1971).

Ferredoxin is the coenzyme that acts as electron receiver. The breakdown of pyruvate results in the production of acetyl-CoA and either reduced ferredoxin (Fd_{red}) in the case of *Clostridia* (reaction 6) or formate in the case of enteric bacteria (reaction 7).

$$\text{Pyruvate} + \text{CoA} + Fd_{ox} \leftrightarrow \text{acetylCoA} + CO_2 + Fd_{red} \quad (6)$$

$$\text{Pyruvate CoA} \leftrightarrow \text{acetylCoA} + \text{formate} \quad (7)$$

In both cases, acetyl-CoA can be further metabolized to acetate or butyrate.

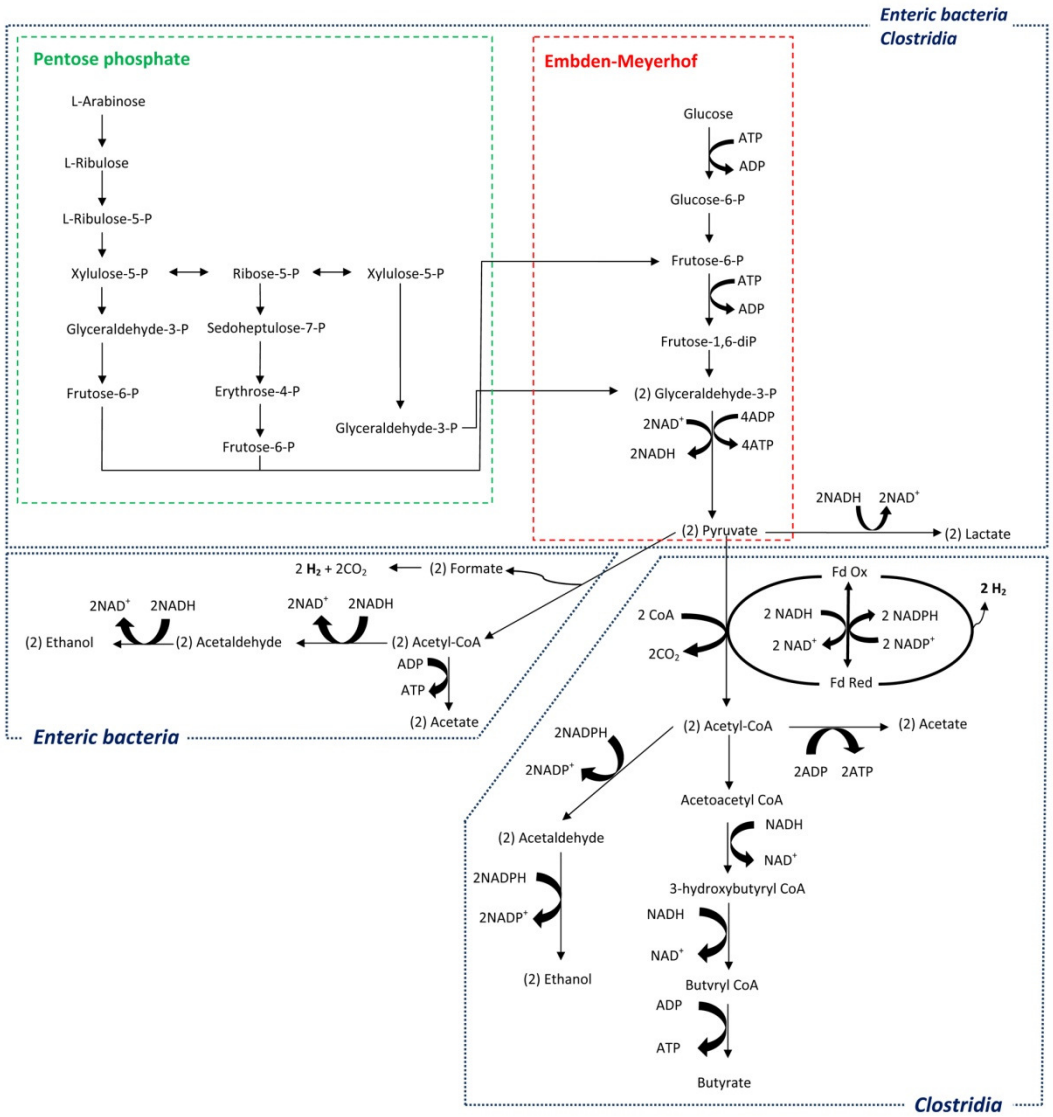

Figure 2.2. – Major catabolic pathways involved in glucose and arabinose fermentation in mixed cultures (adapted from Jones and Woods, 1986; Turcot et al., 2008).

From the re-oxidation of each mole of ferredoxin, one mole of hydrogen will be generated via a hydrogenase enzyme. When acetate is the final product, one extra mol of hydrogen will also be formed from the oxidation of each mole of NADH that was produced during glycolysis. This leads to a total hydrogen yield of 4 moles per mol of glucose consumed. When butyrate is the final product, the NADH produced during glycolysis is used for oxidation of acetoacetylCoA to butyrate and thus the total final hydrogen yield is 2 mol per mol of glucose consumed.

The breakdown of acetyl-CoA allows the maintenance of cell growth, while

generating a variety of fermentation end products. Acetyl-CoA is used to obtain ATP (acetate production) via substrate level phosphorylation, and to the regeneration of NAD^+ needed to maintain glycolysis (e.g. ethanol or butanol production), or for both processes (butyrate, propionate, succinate production) (Temudo et al., 2007). An alternative way to regenerate NAD^+ occurs via lactate formation directly from pyruvate (Hallenbeck, 2005; Temudo et al., 2007).

2.3 Hydrogenases

The core of any biological process leading to hydrogen production is the enzyme hydrogenase (H_2ase). Hydrogenase is surprisingly complex considering that it carries out what is one of the simplest chemical reaction, a reversible oxidation of hydrogen into its elementary particle constituents, two protons (H^+) and two electrons:

$$H_2 \leftrightarrow 2H^+ + 2e^- \qquad (8)$$

Hydrogenases can be classified into two main groups with respect to the metals present in the active sites: those containing only Fe called Fe-H_2ase and those with Ni, Fe and sometimes Se, [Ni-Fe] H_2ase and [Ni-Fe-Se] H_2ase (Vignais et al., 2001). Fe-H_2ase functions either in the utilization of hydrogen as a growth substrate (H_2 uptake) or in the disposing of excess electrons by combining them with protons to form hydrogen (H_2 production). The function of the cytoplasmic enzyme is to remove excess reducing equivalents produced during fermentations carried out by strict anaerobic bacteria and that of the periplasmic enzymes is normally hydrogen oxidation (Peters et al., 1998; Pierik et al., 1998). [Ni-Fe] H_2ase generally functions as an uptake hydrogenase that provides reducing power via hydrogen oxidation (Hallenbeck, 2002)

Several electron carriers have been implicated in the reduction of hydrogenase (i.e. in the direction of hydrogen evolution) including ferredoxin and perhaps flavodoxin. NADH can also serve to reduce hydrogenase in some cases through the action of NADH: ferredoxin oxiredutase. Hydrogen oxidation is coupled to the reaction of electron acceptors such as oxygen, nitrate, sulphate, carbon

dioxide and fumarate (Hallenbeck, 2005).

Environmental factors such as pH, temperature and iron concentration have a direct influence on the activity of the enzyme in the hydrogen production process. In general, hydrogenase activity (uptake and evolution) increases steadily with decreasing pH, until an optimum value at pH of 6.3 (Adams and Mortenson, 1984). The activity of the enzyme is generally low in cells maintained at pH<5.2 (George and Chen, 1983).

In different systems (reactors using soluble and solid substrates), it was observed that the specific hydrogen production rate and hydrogen percentage increased with the temperature and the optimum hydrogen production rate was achieved at thermophilic conditions (Shin et al., 2004; Valdez-Vazquez et al., 2005).

Iron concentration seems to have an important effect on hydrogenase activity (Hawkes et al., 2002). Several studies reported that iron-limited conditions would not only lower the production of hydrogen and acids, but also increase the production of alcohols, such as ethanol and butanol (Lee et al., 2001). However, the reported optimal iron concentration was inconsistent, varying from 10 mg Fe^{2+} per litre (Liu and Shen, 2004) to 353 mg Fe^{2+} per liter (Lee et al., 2001).

2.4 Hydrogen accumulation in anaerobic environments

In most anaerobic environments, hydrogen consumption by different microbial trophic groups proceeds rapidly. Thus, the aim of hydrogen production requires the counteracting of this natural fact. Hydrogen accumulation is therefore linked with the inhibition of hydrogen consuming microorganisms such as hydrogenotrophic methanogens and autotrophic acetogens. These are the main hydrogen-consuming microorganisms when nitrate and sulphate are absent or present at negligible amounts.

There are many options for inhibiting hydrogen-consuming microorganisms, namely by means of environmental pressure, heat-shock treatment and chemical compounds. The selection of an inhibitor method will depend on the initial investment available, the operational costs, technical feasibility and complexity,

inhibition effectiveness during the entire fermentation time, stabilization times of the inoculum, friendliness to hydrogen-producing microorganisms, inocula type and effect on the morphological properties of immobilized inocula (granular sludge).

2.4.1 Environmental pressure

2.4.1.1 Acidic conditions

Under relatively low pH values, methane production stops and H_2/CO_2 are the main gases produced (Phelps and Zeikus, 1984). Most methanogens grow in a relative narrow pH range (6-8). For instance, some species such as *Methanobacterium espanolae* grow at pH between 5.6 and 6.2 but are unable to grow and produce methane at pH 4.7 (Garcia et al., 2000). Low pH (around 5) is effective for inhibiting methanogenesis and obtaining an inoculum rich in hydrogen-producers. Although it is necessary to determine the optimal acidic pH for each inoculum, in general the optimal pH for hydrogen production is between 5 and 6.5 (Lin et al., 2006; Lin and Cheng, 2006). The maintenance of the biomass at this pH range is crucial not only for methanogens inhibition but possibly other hydrogen-consuming microorganisms, such as sulphate reducing bacteria and hydrogen-consuming acetogens (Lowe et al., 1993; RieuLesme et al., 1996). Besides being a control parameter during fermentation, the role of low pH is also linked to the shift of metabolic pathways and hydrogenase activity (see section 2.4 and 2.7.2). The main limitations of this method are the acclimatization time and the eventual presence of hydrogen-consuming microorganisms that tolerate acidic conditions.

2.4.1.2 Short hydraulic retention times

Hydraulic retention time (HRT) is defined as the ratio between the volume of the reactor and the volumetric flow, and is also known as the inverse of the dilution rate (*D*). Only the microbial populations with growth rates larger than the dilution rate ($\mu max > D$) can remain in the reactor. High dilution rates (short HRT) could be used to cause the complete washout of methanogens since the specific

growth rates of methanogens are much shorter than those of hydrogen producing bacteria (indicative values of 0.019 and 0.083 h^{-1}, respectively) (Lema, 1992). The ideal HRT depends on the operational conditions such as substrate, type of inocula and temperature. Recent studies have demonstrated that, usually, at high HRT, the hydrogen production rate is diminished, with a critical value of HRT of 6h (Chen et al., 2001; Han and Shin, 2004). A decrease in HRT can be effective for methanogens washout with suspended sludge systems and using soluble non-complex substrates. In systems with retained microbial biomass, the low HRT method could reveal less effective, since in this kind of processes cell growth is no longer directly controlled by HRT, and slow growing methanogens can flourish even at high-rates of liquid throughput.

2.4.1.3 Heat treatment

Some microorganisms have the capacity to sporulate when environmental conditions become hostile (Foster and Johnstone, 1990). Spores are metabolically dormant and resistant to heat, radiation, desiccation, extreme pH conditions and toxic compounds (Setlow, 2003). In anaerobic environments the main spore forming microorganisms are several genera of acidogenic bacteria, namely *Bacillus*, *Clostridium* and *Thermoanaerobacterium* genera. This fact has been used by several authors to eliminate or kill non-spore-forming microorganisms mainly methanogens, by means of heat treatment of the inoculum. The most common condition applied is to heat the inoculum by boiling (100^{o}C) for 15 to 120 min, although temperatures from 75^{o}C (Chang et al., 2002) to 121^{o}C (Wang et al., 2003) and duration from 15 min (Lay et al., 1999) to 2h (Fan et al., 2004) have been used as well. This treatment simultaneously selects spores of acidogenic bacteria that will germinate, producing hydrogen when the conditions are again favorable for growth. Heat treatment may also prevent the development of non-spore-forming propionate producers which degrade sugars without hydrogen formation (Cohen et al., 1984). Heat treatment has been used by several authors during short operation periods, although increasing evidence shows that a stable hydrogen production and methanogenesis repression is not

possible for long-term continuous operation (Kim et al., 2006b). Furthermore, hydrogen consuming bacteria that can form spores and therefore survive heat treatment, including acetogens (*Acetobacterium*, some *Clostridium* spp., *Sporomusa*), certain propionate and lactate producers (*Propionibacterium*, *Sporolactobacillus*), and sulphate-reducer (*Desulfotomaculum*) will not be eliminated by this treatment (Madigan, 2000).

2.4.2 Chemical inhibitors

Different chemicals have been referred as inhibitors of methane formation by methanogenic archaea. These compounds have different specificities and act at different concentrations. An example of a chemical inhibitor of methanogenesis is 2-bromoethanesulfonate (BES), a structural analogue of coenzyme M (2-mercaptoethanesulfonic acid), the methyl carrier in the final reductive step of methanogenesis (Rouviere and Wolfe, 1988).

Chloroform, fluoroacetate and acetylene are some other examples of inhibitors for methanogens. Chloroform ($CHCl_3$) is known to block the function of corrinoid enzymes and to inhibit methyl-coenzime M reductase (Oremland and Capone, 1988). Chloroform not only inhibits methanogenesis from both H_2/CO_2 and acetate, but also acetate consumption by sulphate-reducers (Chidthaisong and Conrad, 2000). Chidthaisong and Conrad (2000) suggested that chloroform might inhibit hydrogen-dependent homoacetogenesis as well.

Fluoroacetate (FCH_2COO^-) has extensively been used to block acetate metabolism and also inhibits acetoclastic methanogens. The mechanism of inhibition is not yet completely understood. Methanogenesis has been also inhibited by acetylene (C_2H_2). Sprott et al. (1982) found that the intracellular ATP content of methanogens dropped considerably after exposure to C_2H_2.

2.5 Diversity and dynamics of hydrogen producers

Fermentative hydrogen production can be carried out by a wide range of microorganisms, with quite diverse requirements in terms of substrate

preference, pH and temperature (Wang and Wan, 2009). Those parameters do not only determine the growth of the microorganisms but also have a crucial role on the metabolic pathway that will prevail, severely affecting the final observed hydrogen yield. Hydrogen production can be achieved either through the activity of mixed acidogenic microbial cultures, derived from natural environments, or through pure cultures of selected hydrogen producing bacteria. In any case, the final selection of the type of culture to be used (mixed, pure or co-culture), as well as the specific microorganisms, has to be based on the requirements of each process.

2.5.1 Mixed cultures

For full-scale application the use of mixed cultures is considered to be more advantageous, due to the fact that the control and operation of the process is facilitated when no medium sterilization is required, reducing thus the overall cost, whereas it also allows for a broader choice of feedstocks selection (Valdez-Vazquez et al., 2005). The mixed consortia can be derived from a variety of different natural sources, such as sewage sludge (Chang et al., 2002; Noike and Mizuno, 2000), anaerobically digested sludge (Lay, 2001; Oh et al., 2003a), compost (Ueno et al., 2001), animal manure (Yokoyama et al., 2007b) and soil (Van Ginkel et al., 2001).

Despite the advantages of mixed cultures in terms of the process economical viability, their use always implies the possible predominance of non-hydrogen producing species such as methanogens, homoacetogens and lactic acid bacteria, which could eventually lead to process failure. The strategy followed in order to minimize such possibility includes an initial pretreatment of the inoculum and the maintenance of environmental and operational conditions that favor the predominance of the desired hydrogen producing species (see section 2.5). Another possible way to overcome this issue might be the “construction” of a consortia with the goal of creating a community of diverse members, each contributing with a unique and essential metabolic capacity (Brenner et al., 2008).

The total metabolic range of the community would be greater than that of any individual member, while at the same time mutual interdependence would assure stable maintenance of the individual members (Weibel, 2008). However little is known about the complex interactions that occur in natural consortia, or how stable synthetic microbial communities could be built. Thus, additional fundamental work might be required before practically useful synthetic hydrogen production consortia become a reality.

2.5.2 Pure cultures

Alternatively to mixed cultures, many researchers have focused on the use of pure cultures of selected hydrogen producing species. The main arguments for their use are substrate selectivity, easier manipulation of the metabolism by altering growth conditions, higher observed hydrogen yields as an effect of the reduction of undesired byproducts, and the repeatability of the process. However, pure cultures can be very sensitive to contaminations and their use demands, in most of the cases, the presence of aseptic conditions, which significantly increases the overall cost of the process.

Several bacteria are capable of producing hydrogen via dark fermentation (Table 2.2), including strict anaerobes (mostly from *Clostridia* class), facultative anaerobes (*Escherichia coli*, *Enterobacter* sp., *Citrobacter* sp., *Klebsiella pneumoniae*) and also aerobes (*Alcaligenes* sp, *Bacillus* sp.). Among the hydrogen-producing bacteria, *Clostridium* sp. and *Enterobacter* sp. are the most widely studied. Extensive research has also been carried out in hydrogen production at high temperature, using thermophilic and hyperthermophilic bacteria. These included *Caldicellulosirupter saccharolyticus* (van Niel et al., 2002), *Thermoanaerobacterium* sp. such as *T. thermosaccharolyticum* (Thong et al., 2008b) and *Thermatoga* sp. such as *T. maritime* (Schroder et al., 1994) and *T. elfii* (de Vrije et al., 2002). *Halanaerobium saccharolyticum* and *H. lacusrosei*, anaerobic bacteria isolated from the sediments of hypersaline lakes have been described as able to produce fermentative hydrogen from glycerol (Cayol et al., 1994; Cayol et al., 1995; Zhilina et al., 1992). Hypersaline conditions could be advantageous since hydrogen utilizing methane producers are rarely found in

hypersaline environments (Oren, 2001; Oren, 2002), thus avoiding the interference of these archaea on the hydrogen yields.

Table 2.2. - Pure cultures for dark fermentation processes and correspondent hydrogen yields

Microorganism	Maximum hydrogen yield ($molH_2\ mol^{-1}$ hexose)	References
Obligate anaerobes		
Clostridium acetobutylicum	1.8 - 2.0	(Chin et al., 2003; Noike et al., 2002)
Clostridium butyricum CGS5	1.1 – 2.3	(Chen et al., 2005; Noike et al., 2002)
Clostridium pasteurianum	2.08 - 2.4	(Brosseau and Zajic, 1982; Heyndrickx et al., 1990)
Clostridium paraputrificum M21	1.0 – 2.2	(Evvyernie et al., 2000; Evvyernie et al., 2001)
Clostridium thermocellum 27405	2.3	(Levin et al., 2006)
Clostridium thermocellum	1.05 – 1.6	(Levin et al., 2006; Sparling et al., 1997)
Clostridium thermolacticum	1.1 – 1.5	(Collet et al., 2004)
Clostridium diolis	ND	(Matsumoto and Nishimura, 2007)
Clostridium tyrobutyricum	1.47	(Lin et al., 2007)
Clostridium beijerinckii	1.96 – 2.81	(Lin et al., 2007)
Caldicellulosiruptor saccharolyticus	3.3 – 3.6	(Kadar et al., 2004; van Niel et al., 2002)
Thermoanaerobacterium thermosaccharoyiticum	1.4 – 2.53	(Thong et al., 2008b; Ueno et al., 2001)
Thermococcus kodakaraensis	ND	(Kanai et al., 2005)
Thermotoga elfii	3.3	(de Vrije et al., 2002; van Niel et al., 2002)
Ruminococcus albus	0.59 – 2.52	(Ntaikou et al., 2008)
Thermotoga maritima	1.56 - 4	(Nguyen et al., 2008)
Facultative anaerobes		
Enterobacter aerogenes	0.4 – 1.7	(Fabiano and Perego, 2002; Nakashimada et al., 2002)
Enterobacter cloacae	2.1 – 3.4	(Kumar et al., 2001; Kumar and Das, 2001)
Escherichia coli	0.75 – 2.55	(Turcot et al., 2008; Yoshida et al., 2005)
Klebsiella pneumoniae	ND	(Solomon et al., 1995)
Citrobacter amalonaticus	1.12 – 1.24	(Oh et al., 2008)
Citrobacter intermedius	1.0 – 1.5	(Brosseau et al., 1982)
Aerobes		
Alcaligenes eutrophus	ND	(Kuhn et al., 1984)
Bacillus licheniformes	0.34 – 1.04	(Kumar et al., 1995)
Bacillus coagulans	2.28	(Kotay and Das, 2007)

2.5.3 Genetically modified microorganisms

Fermentative hydrogen production processes, either conducted via mixed or pure cultures, still have limitations that can be crucial for scaling up, namely inefficient substrate conversion, formation of byproducts, low resistance to high hydrogen partial pressures and oxygen. In addition, the limiting upper value of hydrogen yield is 4 molH_2 per mol of hexose consumed, despite the fact that one mol of hexose contains 12 gram-atoms of hydrogen. In order to suppress these limitations and enhance the process, recent works proposed the use of genetically modified microorganisms, constructed either via mutagenesis or through genetic engineering. The main areas of modification are summarized as follow: (a) introduction or manipulation of genes responsible for the overexpression of enzymes with cellulolytic activities, such as cellulases, hemicellulases and lignases, or enzymes for uptake of different types of sugars, both aiming at the maximization of substrate availability and conversion; (b) elimination of hydrogen-consuming hydrogenases, and (c) overexpression of hydrogen-producing hydrogenases, that have also been modified to be hydrogen tolerant (Nath and Das, 2004). The bacterium *E. coli* is one of mostly studied facultative anaerobes for hydrogen generation, and has also been widely used as carrier of different genes through mutagenesis and genetic engineering. The construction of many different genetically modified strains has been reported, including strains capable of consuming pentoses (Ghosh and Hallenbeck, 2009), strains with suppressed lactate and succinate dehydrogenases activities (Yoshida et al., 2005) and strains showing overexpression of formate hydrogenlyase (Sanchez-Torres et al., 2009). Increase of cellulolytic activity for enhancing hydrogen production from lignocellulosic biomass has been achieved for *Clostridium beijerinckii* (Claassen et al., 1999).

2.6 Factors/parameters influencing the activity of hydrogen-producing bacteria in anaerobic reactors

2.6.1 Temperature

Temperature is one of the most important parameters that influence the activity of hydrogen-producing bacteria and fermentative hydrogen production. Dark fermentation metabolism takes place in a wide temperature range. Almost all studies of hydrogen production with mixed cultures, have been performed under mesophilic (26-40ºC) or thermophilic (45-60ºC) conditions. Recently, fermentation under extreme-thermophilic conditions (70ºC) started to attract researcher's attention. Besides the more extensive destruction of pathogens and lower risk of contamination by methanogenic archaea (van Groenestijn et al., 2002), biohydrogen fermentation at extreme thermophilic temperatures (over 70^{o}C) has been recognized as thermodynamically advantageous over mesophilic fermentation. Examining the equation, $\Delta G^0 = \Delta H^0 - T.\Delta S^0$, the reaction should became more favorable as the temperature is increased due to the enhancement of the entropy term. Also, at higher temperatures the effect of hydrogen partial pressure is less significant. At 25 °C, the PH_2 needs to be as low as 0.022 kPa to make the reaction (1) feasible, in contrast to only 2.2 kPa at 100 °C (Verhaart et al., 2010). Moreover, other studies at thermophilic conditions have demonstrated higher hydrolysis rate (Lu et al., 2008), and higher H_2 yield (Kadar et al., 2004). The operating costs at higher temperatures will depend largely on the heat exchange efficiency of the plant and insulation of reactors.

2.6.2 pH and end products

The influence of pH has been recognized as a key factor in determining the outcome of hydrogen fermentation. The pH is related with three important features: (a) methanogenic growth limitation (section 2.4.1.1); (b) hydrogen production performance (section 2.3); and (c) regulation of shift to solventogenesis. A pH decrease (by acids accumulation), typically to 4.5, can induce the shift to solventogenesis resulting in a decrease of hydrogen

production (Jones and Woods, 1986).

Acid metabolic end-products are toxic to the microbial cells (Van Ginkel and Logan, 2005). Inhibition is caused by nonpolar undissociated acids being able to cross the cell membrane at a low pH, that then dissociate into the cell at the higher internal pH, releasing protons inside the cell. The uptake of protons in this way uncouples the proton motive force, which causes an increase in maintenance energy requirements to maintain the intracellular pH near neutrality. When the concentration of the undissociated acids becomes sufficiently high, the pH gradient across the membrane collapses, which results in the total inhibition of all metabolic functions in the cell. The shift to solventogenesis has been related to a detoxification mechanism of the cell to avoid these inhibitory effects (Jones and Woods, 1986).

2.6.3 Hydrogen partial pressure

The metabolic pathways of hydrogen formation are sensitive to hydrogen concentrations and are subject to end-product inhibition. Therefore, the hydrogen partial pressure (PH_2) is an extremely important factor for continuous hydrogen synthesis. The re-oxidation of reduced ferredoxin and hydrogen carrying enzymes becomes less favorable when hydrogen concentration in the liquid increases (Hawkes et al., 2002; Stams, 1994). Consequently, hydrogen production decreases and the metabolic pathways shift to production of more reduced substrates such as lactate, ethanol, acetone, butanol, or alanine (Levin et al., 2004).

Several strategies have been developed to avoid the negative effect of hydrogen accumulation. These include vigorous mixing to avoid supersaturation (Lay, 2000), sparging with inert nitrogen (Hussy et al., 2005; Mizuno et al., 2000a), utilization of hydrogen permeable membrane to remove dissolved hydrogen from mixed liquor (Liang et al., 2002) and continuous pressure release (Logan et al., 2002). The sparging with nitrogen has the disadvantage of diluting hydrogen content in the biogas, resulting in a cost increase for its recovery.

2.6.4 Feedstocks

Theoretically any organic substrate rich in carbohydrates, fats and proteins could be considered as possible substrate for biohydrogen production. However, carbohydrates are the main source of hydrogen during fermentative processes and therefore waste and biomass rich in sugars and/or complex carbohydrates turn out to be the most suitable feedstocks for biohydrogen generation (Kapdan and Kargi, 2006). The major criteria that have to be met for the selection of substrates suitable for fermentative biohydrogen production are availability, cost, carbohydrate content, biodegradability and concentration of inhibitory compounds (Hawkes et al., 2002).

Simple sugars such as glucose, xylose, arabinose, sucrose and lactose are readily biodegradable and thus preferred as model substrates. Hydrogen production has been demonstrated also from energy crops, wastes and residues (Table 2.3). Energy crops could be quite sufficient for hydrogen generation (de Vrije et al., 2009; Ntaikou et al., 2008), however the continuously rising food prices, the sustainability doubts and the energy equation challenges have led to a reaction against the use of energy crops as feedstocks for biofuel generation. An alternative is the production of second generation biofuels using feedstocks that are not competitive to edible crops, such as wastes and residues (Ntaikou et al., 2010).

Different types of lingocellulosic residues have been studied as potential renewable feedstocks for fermentative hydrogen production (Table 2.3). These include agricultural residues, such as sugar cane and sweet sorghum bagasse, corn stalks and stover, fodder maize, wheat straw, etc., and forestry residues, such as wood trimmings. Compared to the use of energy crops, the exploitation of residues, remaining after the harvesting and processing of the starch or sugar crops that cannot be further exploited in the food industry chain, is more likely to yield a solution with far better overall prospects for economic and environmental sustainability (Lynd et al., 2005).

Table 2.3. - Comparison of various substrates used for fermentative hydrogen production

Inoculum	Carbon source	H_2 production rate	H_2Yield	Reference
Municipal sewage sludge	Xylose (20g COD/L)		2.25 molH_2/mol xylose	(Lin and Cheng, 2006)
Mixed culture	Glucose		2.8 mol H_2/mol glucose	(Luo et al., 2008))
Mixed culture	Glucose (1g COD/L)		0.9 mol H_2/mol glucose	(Logan et al., 2002)
C. pasteurium	Sucrose (20g COD/L)	252 mL H_2/L.h*	4.8 mol H_2/mol sucrose	(Lin and Chang, 2004)
Mixed culture	Sucrose (1g COD/L)		1.8 mol H_2/mol sucrose	(Logan et al., 2002)
Thermoanaerobacterium	Starch (4.6 g/L)	1.9 mL H_2/h	92 mLH_2/g starch	(Zhang et al., 2003)
C. pasteurium	Starch (24 g/L)	4.2 mL H_2/h	106 mLH_2/g starch	(Liu and Shen, 2004)
Cow dung compost	Cornstalk wastes		150 mLH_2/TVS	(Zhang et al., 2007a)
Anaerobic digested sludge	Rice slurry		346 mL H_2/g carbohydrate	(Fang et al., 2006)
Cow dung compost	Beer lees		68.6 mLH_2/TVS	(Fan et al., 2006a)
Fermented soybean-meal	Bean curd	130 mL H_2/h.L culture		(Mizuno et al., 2000b)
Anaerobic sludge	Food waste		1.8 mol H_2/mol hexose	(Shin et al., 2004)
Mixed culture	Food waste (3% VSS)		111 mL H_2/gVSS	(Kim et al., 2004)
Activated sludge	Food wastewater		47.1 mmol H_2/gCOD	(Wu and Lin, 2004)
Mixed culture	Rice winery wastewater	159 mLH_2/L.h	2.14 mol H_2/mol hexose	(Yu et al., 2002)

Table 2.3 – Continued

Inoculum	Carbon source	H_2 production rate	Yield	Reference
Mixed culture	Domestic wastewater		0.01L H_2/L WW	(Van Ginkel et al., 2005)
Mixed culture	Molasses	200 mL H_2/L.h		(Ren et al., 2007)
C. butyricum + *E. aerogens*	Sweet potato starch (2%)		2.7 molH_2/mol glucose	(Yokoi et al., 2002)
Mixed culture	Olive pulp	13 mL H_2/L.h*		(Gavala et al., 2005)
Mixed culture	Sugar factory wastewater	180 mL H_2/L.h	2.6 molH_2/mol hexose	(Ueno et al., 1996)
Rumicococcus albus	Sweet sorghum stalks		59 L H_2/kg wet biomass	(Ntaikou et al., 2008)
Caldicellulosiruptor saccharolyticus	Sweet sorghum		30.17 L H_2/kg dry biomass	(Ivanova et al., 2009)
Mixed culture	Corn starch	110 mLH_2/L.h	0.51 mol H_2/mol hexose	(Arooj et al., 2008)
Clostridium thermocellum	Wood fibers		1.47 mol H_2/mol hexose	(Levin et al., 2006)
Caldicellulosiruptor saccharolyticus	Wheat straw		44.7 LH_2/kg dry biomass	(Ivanova et al., 2009)
Caldicellulosiruptor saccharolyticus	Maize leaves		31.5 LH_2/kg dry biomass	(Ivanova et al., 2009)
Mixed culture	Cheese whey	100 mLH_2/L.h	0.9 mol H_2/mol hexose	(Venetsaneas et al., 2009)

*Calculated from the data at standart temperature and pressure (STP)

Although being an abundant and almost zero cost feedstock, agricultural and forestry residues do not contain easily fermentable free sugars, but complex carbohydrate polymers, i.e. cellulose and hemicellulose, which are tightly bonded to lignin.

Several food processing industrial wastewaters can be considered suitable feedstocks for hydrogen production via dark fermentation (Table 2.3). Rice winery, noodle, sugar, and molasses manufacturing, olive mill wastewater, olive pulp, and cheese whey are some of the wastewaters that have been successfully tested for hydrogen production at laboratory scale. Complex solid wastes, such as the ones from kitchen, food processing, mixed wastes, and municipal wastes have also been tested. In general, apart from carbohydrates, these wastes have relatively high content of proteins and fats, and thus their conversion efficiencies to hydrogen are comparatively lower than those obtained from carbohydrate based wastewaters (Lay et al., 2003).

Among different types of wastes, the organic fraction of municipal solid waste (OFMSW) may be considered quite promising as potential feedstock for hydrogen production, since it can represent up to 70% of the total MSW produced, consisting of paper (up to 40%), garden residues, food wastes and wood (Ntaikou et al., 2010). Another waste that is attracting increasing attention is crude glycerol, a by-product from the biodiesel production industry (Ito et al., 2005). The recent increase of biodiesel production from vegetable oils and fats has lead to the generation of large quantities of glycerol that have to be disposed.

2.6.5 Nutrients requirement

Like all fermentation processes, hydrogen production requires nutrients for bacterial metabolism, growth and activity. The nutrients include nitrogen, phosphate and some trace elements.

Nitrogen is one of the most essential nutrients for growth. Comparisons of hydrogen yield at various nitrogen concentrations and C/N ratios were reported. Results were, however, conflicting. The optimum nitrogen concentration varied

from 0.1 to 2 g N L^{-1} and C/N ratio from 3.3 to 130 (Lin and Lay, 2004a; Liu and Shen, 2004; Ueno et al., 2001).

Phosphate was also reported as necessary in hydrogen production, due to its nutritious value, as well as buffering capacity (Lin and Lay, 2004c; Oh et al., 2003b). Hawkes et al. (2002) compared literature data for C/P ratios from 6 to 260 and referred an optimal C/P ratio of 130. A similar optimal C/P ratio of 120 was reported based on a systematic study for C/P ratios from 8.7 to 800 for hydrogen production from sucrose (Lin and Lay, 2004b).

In terms of trace elements, it was reported that magnesium, sodium, zinc and iron were relevant for hydrogen production. Furthermore, among the four elements, magnesium was the most crucial. At optimal combined concentrations of 4.8 mg Mg^{2+} L^{-1}, 393 mg Na^{+} L^{-1}, 0.25 mg Zn^{2+} L^{-1}, and 1 mg Fe^{2+} L^{-1}, the maximum hydrogen yield from a sucrose containing wastewater was 233 mLH_2 g^{-1} hexose (Lin and Lay, 2005).

2.6.6 Toxicity and inhibition

Heavy metals, including cadmium, chromium, zinc, copper, nickel, and lead may be present at significant concentration in some industrial wastewater and municipal wastes. These metals are often found to be the leading cause of anaerobic reactor upset and failure. Fang and Chan, (1997) compared the effect of heavy metals on the methanogenic granular sludge, and reported that toxicity was in the following order: zinc > nickel > copper > cadmium > chromium. For hydrogen production, similar amplitude of inhibition was also observed, as shown by IC_{50} values (concentration of inhibitor that causes 50% relative activity loss). Lin and Shei, (2008) reported that for hydrogen production from sucrose, zinc (IC_{50}=4.5 mg L^{-1}) was slightly more toxic than copper (IC_{50}= 6.5 mg L^{-1}), which in turn was much more toxic than chromium (IC_{50}=60 mg L^{-1}). Zheng and Yu, (2004) reported that there was a lag phase for hydrogen production from glucose in the presence of copper and zinc. However, the total hydrogen yield was not adversely affected by copper at concentrations up to 400 mg L^{-1} and by zinc up to 500 mg L^{-1}.

During physicochemical or biological pretreatment of lignocellulosic biomass the generation of undesirable organics substances usually occurs, which may be inhibitory to the microbial metabolism (Pattra et al., 2008; Persson et al., 2002). The most common are furfural (FF, $C_5H_4O_2$) and 5-hydroxymethylfurfural (HMF, $C_6H_6O_3$), which are formed during the thermal decomposition of sugars and carbohydrates, and phenolic compounds produced during the degradation of lignin. During thermochemical pretreatment, all three inhibitory compounds are formed, whereas during biological pretreatment only phenolic compounds emerge. The severity of the inhibitory effects depend on the concentrations and thus, in order to achieve efficient fermentations, either mild pretreatment methods should be selected or the inhibitory compounds should be removed prior to feeding the fermentation bioreactors (Pattra et al., 2008; Persson et al., 2002). Although the inhibitory effect of FF, HMF and phenolic compounds on hydrogen producing bacteria is not yet well defined, it is expected that the results would be similar to those observed during fermentations for ethanol or methane production.

Hydrogen production may also be inhibited by the presence of certain bacteria. Noike et al. (2002) reported that hydrogen production by *Clostridium* ceased when two lactic acid bacteria, *Lactobacillus paracasei* and *Enterococcus durans* were added as co-cultures. These authors claimed that the inhibition was caused by bacteriocins secreted by the lactic acid bacteria, and not by the acidification of the medium. Other authors reported also the inhibitory effect of bacteriocins secreted by *Lactobacillus plantarum* on the growth of *L. casei, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsielia pneumoniae* and *Escherichia coli*.

2.7 Type of reactors

Reactor configuration is considered to be crucial for the overall performance of fermentative hydrogen production. It influences the reactor microenvironment, the prevailing microbial population, the established hydrodynamic behaviour and the contact between substrate and consortia (Mohan, 2009). In general, mixed

reactors for fermentative hydrogen production can operated in batch or continuous mode. Batch mode fermentative hydrogen production is suitable for research proposes (Lee et al., 2002), but any industrially feasible process would most likely have to be performed in continuous or at least semi-continuous (fed or sequencing batch) basis.

Continuous processes for fermentative hydrogen production can be performed in suspended or immobilized systems (Oh et al., 2004) (Table 2.4). Suspended systems allow better mass transfer between microorganisms and substrates, but the washout of H_2-producing bacteria under low hydraulic retention time is more likely to occur (Chen et al., 2001). The Continuously Stirred Tank Reactor (CSTR) is the most commonly used reactor for suspended systems, offering simple construction, ease of operation and effective homogenous mixing, as well as temperature and pH control. In CSTR, biomass has the same retention time (SRT) as the HRT, and thus, its concentration in the mixed liquor is low, limiting the rate of hydrogen production.

Recently, immobilized based systems have been used for hydrogen production since they are capable of maintaining higher biomass concentration under high dilution rates, reducing biomass washout (Zhang et al., 2008). In general, immobilized-cell techniques include surface attachment (Oh et al., 2004), self-flocculation (Kim et al., 2005), gel entrapment (Wu et al., 2006) and retentive membranes (Hallenbeck and Ghosh, 2009).

These techniques have been applied to produce H_2 continuously in fixed or packed-bed bioreactors (Lee et al., 2003), granular sludge bed bioreactors (e.g., carrier induced granular sludge bed (CIGSB)) (Lee et al., 2004), trickling biofilter reactors (TBR) (Oh et al., 2004), up-flow anaerobic sludge blanket bioreactor (UASB) (Thong et al., 2008a), fluidized bed bioreactors (FBR) (Lin and Cheng, 2006) (see Table 2.4).

A direct comparison of the performance of different types of reactor configurations that have been studied in terms of hydrogen productivity is not possible, since the operational parameters (feedstocks and operating conditions) along with the reactor configuration is, in all studies, quite different.

Table 2.4. – Reactor configurations for dark fermentation (adapted from Hallenbeck and Ghosh, 2009)

Type of reactor	Substrate	Inoculum	Temperature (^{o}C)	H_2 production rate ($LH_2\ L^{-1}\ h^{-1}$)	Reference
Continuously Stirred Tank Reactor (CSTR)	Molasses	Sludge	30	0.20	Ren et al, 2007
Anaerobic sequencing batch reactor (ASBR)	Glucose	Sludge	34.5	0.23	Cheong et al., 2007
Fixed bed bioreactor with activated carbon (FBBAC)	Sucrose	Sludge	35	1.2	Chang et al., 2002
Anaerobic fluidized bed reactor (AFBR)	Glucose	Sludge	37	2.4	Zang et al., 2007b
Upflow anaerobic sludge blanket reactor (UASB)	Sucrose	Sludge	35	0.27	Chang et al., 2004
Carrier-induced granular sludge bed (CIGSB)	Sucrose	Sludge	35	9.3	Lee et al., 2006
Fluidized bed reactor (FBR)	Sucrose	Sludge	35	1.4	Wu et al., 2007
Anaerobic fluidized bed reactor (AFBR)	Glucose	Sludge	37	7.6 biofilm reactor; 6.6 granule reactor	Zhang et al, 2008
Membrane bioreactor (MBR)	Glucose	Heat treated soil	not referred	0.38	Oh et al., 2004

2.8 Second stage processes

Fermentative hydrogen production processes are not effective in terms of chemical oxygen demand (COD) removal. Further utilization of the organic matter contained in the effluent of a fermentative hydrogen producing reactor could increase the overall energy output of the process and/or more products recovery. The development of a two stage process, so far, involves the fermentation of the substrate to hydrogen and organic acids in the first stage and, in a second stage, either an additional energy extraction or the generation of high added value products by exploiting the effluent of the first stage reactor (Figure 2.3).

One of the second stage processes coupled to fermentative hydrogen production is photofermentation. This approach increases the overall energy extraction, with the recovery of additional hydrogen. In such a two-stage process, the effluent rich in organic acids produced in the first stage, by fermentative bacteria, can be converted to hydrogen, in the second step, by photosynthetic bacteria which capture and use of light energy (Chen et al., 2008; Nath et al., 2008).

Another approach used for further utilization of the remaining organic matter is to produce, in a second stage, an additional useable energy carrier such as methane. A two stage process technique, combining acidogenesis and methanogesis appears to give more efficient waste treatment and energy recovery than a single methanogenic process (Hawkes et al., 2007). Recently, it was also found that the addition of hydrogen up to 10% to the biogas (CH_4 + CO_2) enhances the combustion characteristics of biogas and reduces emissions. This specification, called biohythane, was found to be most suitable for burning directly in the internal combustion engines (Porpatham et al., 2007).

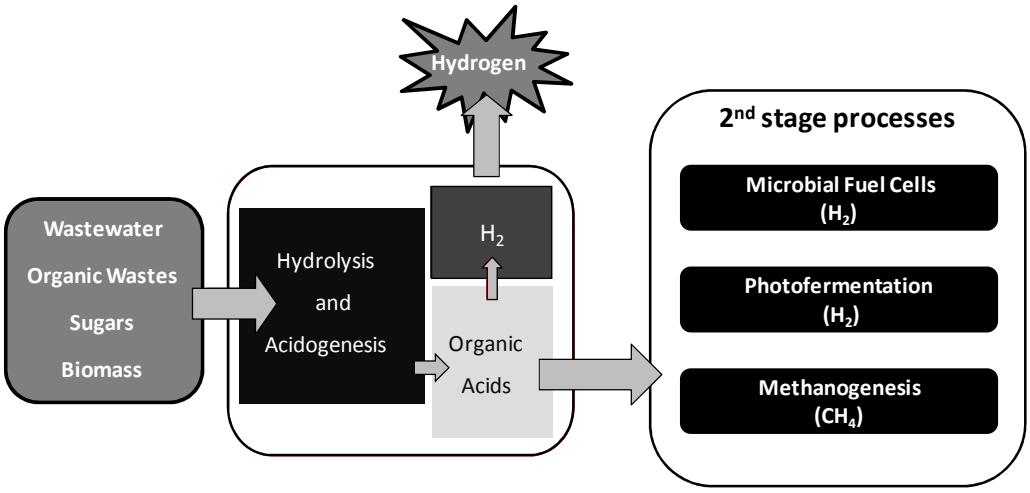

Figure 2.3. – Schematic representation of the combined processes of dark fermentative biohydrogen production and second stage processes.

Microbial fuel cells (MFC) can utilize acetate and butyrate to produce electricity (Liu et al., 2005a). Oh and Logan (2005) demonstrated that the fermentation end-products of batch hydrogen production from a cereal processing wastewater high in sugar could be used by a MFC to produce electricity with 95% COD removal. MFC technology is currently at the research stage and still needs development (Rabaey and Verstraete, 2005).

A recently reported option for enhancing biohydrogen recovery is the bio-electrochemically assisted microbial reactor (BEAMR) with low voltage conversion of acetate to H_2 (Liu et al., 2005b; Rozendal et al., 2006). BEAMR technology is a modified application of microbial fuel cells with an additional small electric potential applied. It allows another process configuration in which additional hydrogen can be produced from fermentation end products, possibly with effluent then passing to a MFC to generate electricity. Either enhanced hydrogen production or electricity generation may prove to be an attractive option for the second stage as these technologies develop further.

An alternative approach is to use the acids rich effluent from dark fermentation to produce other high added value products such as polyhydroxyalkanoates (PHAs), via selected microorganisms (Ntaikou et al., 2009).

2.9 Conclusions and perspectives

In the preceding sections, fundamental information about dark-fermentative biohydrogen production was presented, as well as the main problems and challenges associated with this process. Fermentative biohydrogen production is characterized by relatively high production rates but low substrate conversion efficiencies. The low hydrogen yields come from the fact that carbohydrates are not fully metabolized, leading to incomplete conversion to H_2 and CO_2 and also from the generation of reduced byproducts, such as lactic acid and alcohols.

Important improvements have been made by manipulation and optimization of bioprocess parameters as well as with the choice of organisms used. The identification of suitable feedstocks for fermentative biohydrogen production has been done, but more research is necessary in this area to improve hydrogen production rates and yields. The use of other approaches, like metabolic engineering and various two stage processes are also been actively investigated in an attempt to solve this problem.

Possible improvements to biohydrogen production may be achieved through specialized bioreactor configuration that might improve biomass concentration and substrate conversion efficiency. This will lead to systems with more robust and reliable performances that will be stable over long periods of time and resistant to short-term fluctuations in operational parameters. In addition, optimized volumetric production rates could also be obtained. Most of the reactors that allow biomass retention depend upon the formation of flocs or granules and more research in this topic is also needed.

The last call launch by the European Union in the framework of the fuel cell and hydrogen joint undertaken, specifically the call related to low temperature hydrogen production processes (SP1-JTI-FCH.2010.2.4), defined expected outcomes that included biological hydrogen production processes based on second generation biomass feedstock or production of hydrogen utilizing different waste biomass focusing on those that allow a sufficient productivity (1-10 kg H_2/d).

Presently, fermentative biohydrogen technology is not ready for industrial application. Its future as alternative process for hydrogen production will depend on the efforts applied to research and development of this process, in the next years.

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Effect of arabinose concentration on dark fermentation hydrogen production using different mixed cultures

Chapter 3

Abstract

Dark fermentation hydrogen production from arabinose at concentrations ranging between 0 and 100 g L^{-1} was examined in batch assays for three different mixed anaerobic cultures, two suspended sludges (S1, S2) obtained from two different sludge digesters and one granular sludge (G) obtained from a brewery wastewater treatment plant. After elimination of the methanogenic activity by heat treatment, all mixed cultures produced hydrogen, and optimal hydrogen rates and yields were generally observed for concentrations between 10 and 40 g L^{-1} of substrate. Higher concentrations of arabinose up to 100 g L^{-1} inhibited hydrogen production, although the effect was different from inoculum to inoculum. It was evident that the granular biomass was less affected by increased initial arabinose concentrations when calculating the rate of decrease in hydrogen yields versus arabinose concentrations, compared against the two suspended sludges. The largest amount of soluble microbial product produced for all three inocula was for n-butyrate. Also, valeric acid production was observed in some samples.

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3.1 Introduction

Hydrogen appears to be an ideal candidate as an alternative to fossil fuels. It has the highest energy content per unit of weight for any known fuel, it is fifty percent more efficient than gasoline in automobiles, and it can be used to generate electricity by fuel cell technology (Moore and Raman, 1998; Sen Gupta et al., 2005). Hydrogen can be obtained via non-biological and biological processes. Non-biological processes use fossil fuels as a source for hydrogen production (Das and Veziroglu, 2001). In this case, however, hydrogen cannot be considered an alternative energy source. Conversely, hydrogen can be obtained biologically from photolysis carried out by algae and cyanobacteria and also via fermentation by anaerobic bacteria. However, the rate of hydrogen production from fermentation is greater compared to photolysis (Das and Veziroglu, 2001).

Dark fermentation of hexoses has been extensively studied using a variety of anaerobic inocula under different growth and operational conditions while biohydrogen production from pentoses has been less well characterized (Li and Fang, 2007). Few reports have demonstrated biohydrogen production directly from arabinose, one of the most common pentoses and a component of various hemicellulosic and plant polysaccharides. Two studies have successfully resulted in the isolation of *Clostridia* species that produced hydrogen using arabinose as the substrate (Taguchi et al., 1993; Taguchi et al., 1994). However, the effect of substrate concentration on hydrogen production was not determined and the products of arabinose fermentation were not identified.

Previous studies carried out with other sugars have shown that different substrate concentrations have an effect on the amount of hydrogen produced (Fang et al., 2006; Lin and Cheng, 2006; Van Ginkel et al., 2001; Wu et al., 2006). In addition, different sources of inocula may also lead to different yields of hydrogen with varying production rates (Li and Fang, 2007; Lin et al., 2006; Van Ginkel et al., 2001). The work presented herein examines the effect of different

concentrations of arabinose on hydrogen production for three different anaerobic mixed cultures.

3.2 Material and Methods

Inocula

Anaerobic sludge was obtained from three different wastewater treatment plants in Portugal. Sludge S1 was dispersed sludge obtained from a sludge digester supplemented with fat, located in a municipal wastewater treatment plant in Coimbra. Sludge S2 was dispersed sludge obtained from a municipal wastewater treatment plant digester located in Oporto. Sludge G was obtained from an upflow anaerobic granular sludge (UASB) reactor treating brewery wastewater. Sludges S1, S2 and G were heat treated in order to suppress the methanogenic hydrogenotrophic activity.

Batch culture inoculation and operation

Batch experiments were conducted in 125 mL serum bottles containing 20 mL total of inocula and media. The media composition was as previously described (Zehnder et al., 1980). The initial biomass concentration was approximately 10 g L^{-1} of volatile suspended solids.

Prior to inoculation, suspended heat treated sludge was centrifuged (5,000 rpm for 5 min), washed in media, centrifuged (5,000 rpm for 5 min), and added to serum bottles. Heat treated granular sludge was first filtered using a 0.2 mm sieve. Then, the sludge remaining on top of the sieve was added to serum bottles. The final concentration of L-arabinose in each bottle was 0, 10, 20, 30, 40, 50, 75, and 100 g L^{-1}. The initial pH of the batch experiments was adjusted to 6.5 by flushing the headspace of each batch reactor with 100% CO_2 for several minutes. Batch cultures were placed on a rotary shaker (150 rpm) and incubated at 37 ºC (± 2 ºC). Experiments at each substrate concentration were conducted in triplicate.

Monitoring and Analysis

Soluble fermentation products (formate, acetate, propionate, *n*- and *i*-butyrate, valerate, and ethanol) and arabinose were determined using high performance liquid chromatography (Jasco, Japan) with a Chrompack column (6.5 x 30 mm^2). Sulfuric acid (0.01 N) was used as the mobile phase at a flow rate of 0.7 mL min^{-1}. The temperature of the column was set at 60 ºC. Detection of VFA, ethanol, and arabinose was accomplished by using a UV detector at 210 nm and a Refraction Index (RI) detector, respectively.

Samples of biogas (0.1 or 0.2 mL) were removed using a gas-tight, gas-locking syringe. Hydrogen concentrations were monitored using a Hayesep Q column (80/100 mesh) and a thermal conductivity detector (Varian 3300 Gas Chromatograph) with nitrogen (30 mL min^{-1}) as the carrier gas. The injector, detector, and column temperatures were 120, 170, and 35 ºC, respectively. Methane concentrations were monitored using a Porapak Q (180 to 100 mesh) column and a thermal conductivity detector (Chrompack), with helium as the carrier gas (30 mL min^{-1}) and having the injector, detector, and oven temperatures set at 110, 110, and 35 ºC, respectively. The quantity of each gas was corrected to 1 atm and 0 ºC. Gas pressure was released using the Owen method (Owen et al., 1979) via a 20-mL or 50 mL glass syringe. The amount of gas present in the headspace of each batch reactor was determined before and after releasing gas pressure. Hydrogen, VFA, and ethanol concentrations for the control inocula (0 g L^{-1} of arabinose) were subtracted from the values obtained in the tests with 10 to 100 g L^{-1} arabinose. Volatile solids and Volatile suspended solids were measured according to Standard Methods (APHA, 1989).

Hydrogen production rates and potential were determined using the Modified Gompertz equation (Equation 1) (Lay et al., 1999; Taguchi et al., 1993; Zwietering et al., 1990):

$$H(t) = P\exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \qquad (1)$$

where H(t) is the cumulative hydrogen production (mL); P is the hydrogen production potential (mL), R_m is the maximum hydrogen production rate (mL h^{-1}); *e* is approximately 2.718, λ is the duration of the lag phase (h), and t is time (h).

3.3 Results and Discussion

Hydrogen production occurred for all three sludges but there were differences in the yields, lag times, and rates. Methane production was not detected in any of the batch cultures. An example of the hydrogen production for the three different inocula for an initial arabinose concentration of 75 g L^{-1} is shown in Figure 3.1.

Granular sludge produced the most hydrogen (50 mL) with the shortest lag phase (15 h) followed by S2 (34 mL and 29 h) with S2 biomass producing the least hydrogen (approximately 15 mL) with the longest lag phase (approximately 45 h). The Modified Gompertz equation was used to calculate the values for the maximum hydrogen production rate, hydrogen production potential, and duration of the lag phase for all batch reactors. In addition, the R^2 values listed are the ranges of the values obtained for modelling the individual triplicate bottles. The results are shown in Table 3.1.

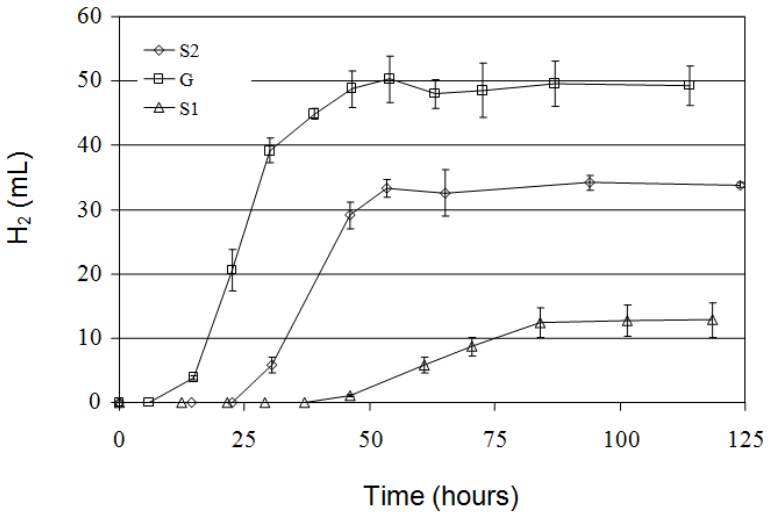

Figure 3.1. Biohydrogen production from three different sludges with an initial arabinose concentration of 75 g L^{-1}. Error bars represent one standard deviation of triplicate bottles.

For the S1 biomass, the largest amount of hydrogen production and the maximum rates were obtained for concentrations of arabinose of 30 and 40 g L^{-1}, respectively. Similar values were also obtained for arabinose concentrations of 20 and 50 g L^{-1}. However, the lag phase was longer for 10 and 20 g L^{-1} arabinose compared to concentrations between 30 and 50 g L^{-1}. The rate of hydrogen production and hydrogen production potential decreases for 75 g L^{-1} and reaches a minimum at 100 g L^{-1}. This suggests an inhibitory effect by high concentrations of arabinose. Previous studies have shown that hydrogen production and rates peak at 20 g L^{-1} COD xylose (another pentose) and decrease significantly when initial concentrations were increased (Lin and Cheng, 2006). Possible reasons for the decrease in hydrogen production include substrate inhibition, product inhibition, a combination of both types of inhibition (Kotay and Das, 2007; Thong et al., 2008b; van Niel et al., 2003). Similar results were observed for the S2 biomass. Maximum hydrogen production did not differ significantly for arabinose concentrations between 10 and 50 g L^{-1}, though peaking at 40 g L^{-1}. The amount of hydrogen production from 75 g L^{-1} arabinose was much higher for S2 than for the S1 sludge. The highest concentration of arabinose tested (100 g L^{-1}) yielded the lowest hydrogen production, lowest rate, and the longest lag time further indicating of the inhibitory effects caused by high concentrations of substrate.

The granular sludge produced similar amounts of hydrogen for concentrations between 10 and 75 g L^{-1}, reaching a peak production of 60 mL of H_2 for 30 g L^{-1} arabinose. It also endured similar lag times with the shortest lag of 10 hr for 20 g L^{-1} arabinose. The lowest amount of hydrogen produced (25 mL) was for 100 g L^{-1} arabinose. Arabinose concentrations of 75 and 100 g L^{-1} generated the largest hydrogen production rates and potentials and shortest lag times when compared against the results from the other two sludges. One possible explanation for the smaller inhibitory effect is the granular nature of the sludge. Hydrogen producing populations just beneath the surface of the granule would be exposed to a substrate concentration gradient that, at decreased concentrations of arabinose or metabolic byproducts, is possibly no longer inhibitory. The high degree of

correlation between the data and the model for all three biomasses suggested that the Modified Gompertz Equation adequately described the data.

Hydrogen yields were calculated for all batch reactors based on the amount of arabinose consumed and the amount of hydrogen produced. Figure 3.2 depicts the changes in the maximum rate of hydrogen production (R_m) and hydrogen yields versus initial arabinose concentrations. In general, as the initial concentration of arabinose increases, hydrogen yields and rates decrease. However, there are differences between the three different biomasses. Significant decreases in yields and rates were observed for the initial arabinose concentrations of 75 and 100 g L^{-1} and 100 g L^{-1} for the S1 and S2 biomasses, respectively. However, for the granular biomass, rates of hydrogen production potential were similar for concentrations between 10 and 100 g L^{-1}. Therefore was evident that the G biomass was less affected by increased initial arabinose concentrations when calculating the rate of decrease in hydrogen yields versus arabinose concentrations (slope=-0.10; R^2 = 0.9946), compared against S2 biomass (slope=-0.16; R^2 = 0.9181) and S1 biomass (slope=-0.19; R^2 = 0.9279) (data not shown).

In addition, the consumption of arabinose decreased for higher concentrations of arabinose suggesting inhibition (Table 3.2). The highest hydrogen yield was observed for the S2 biomass (1.98±0.31 mol H_2/mol substrate consumed) for 10 g L^{-1} arabinose. Granular biomass produced the highest hydrogen yield for 10 g L^{-1} arabinose when compared to other concentrations for the same inoculum. However, the amount (1.56±.01 mol H_2/mol arabinose) was lower than the value for the S2 biomass. The highest hydrogen yield for the S1 sludge was produced at 20 g L^{-1} arabinose and was the smallest of all the inocula tested (1.46±0.09 mol H_2/mol arabinose).

Table 3.1. - Modified Gompertz equation parameters for the three different sludges with varying amounts of arabinose where P = the hydrogen production potential, R_m = maximum hydrogen production rate, and λ = lag phase. The R^2 values listed are the range of the values obtained for modeling the individual triplicate bottles

Arabinose (g L^{-1})	P (mL)	R_m (mL h^{-1})	λ(h)	R^2
Suspended Sludge(S1)				
10	35.9±1.7	1.5±0.5	37.5±1.1	0.9919 - 0.9937
20	53.9±1.3	2.3±0.1	38.2±4.8	0.9669 - 0.9720
30	59.7±4.2	2.8±0.3	16.0±1.5	0.9994 - 0.9996
40	53.6±3.3	2.5±0.0	17.8±0.4	0.9959 - 0.9998
50	49.1±1.1	2.4±0.2	17.5±0.4	0.9964 - 0.9987
75	13.1±2.6	0.4±0.1	47.0±4.3	1.0000 - 1.0000
100	2.9±0.1	0.3±0.0	58.1±0.6	0.9962 - 0.9966
Suspended Sludge (S2)				
10	51.0±6.2	3.5±0.5	20.3±1.9	0.9937 - 1.0000
20	52.9±1.8	2.3±0.2	13.5±6.3	0.9582 - 0.9992
30	46.7±1.2	3.1±0.4	15.5±1.0	0.9375 - 1.0000
40	53.1±2.6	3.8±0.0	19.6±0.5	0.9999 - 1.0000
50	50.8±0.9	3.3±0.8	18.4±1.1	0.9993 - 1.0000
75	33.8±1.8	2.3±0.3	28.7±5.3	0.9996 - 1.0000
100	9.0±1.8	0.4±0.4	87.5±3.8	1.0000 - 1.0000
Granular Sludge (G)				
10	46.8±0.3	3.8±0.3	14.7±0.7	0.9999 - 1.0000
20	48.7±0.4	3.5±0.1	10.8±4.1	0.9983 - 0.9999
30	60.3±0.2	3.6±0.2	13.2±0.4	0.9991 - 0.9999
40	50.6±0.4	2.9±0.4	11.0±1.8	0.9999 - 1.0000
50	52.7±1.8	2.7±0.6	11.5±0.3	0.9926 - 0.9989
75	50.1±4.3	2.9±0.4	14.9±0.4	0.9956 - 0.9998
100	24.9±0.7	2.7±0.0	23.0±0.4	1.0000 - 1.0000

The yields and rates of hydrogen production are different when compared against the values obtained for pure culture *Clostridium* sp. No. 2 fed arabinose (Taguchi et al., 1994). *Clostridium* sp. strain No. 2 produced 3600 mL H_2/L culture with a hydrogen yield of 2.2 mol H_2/mol of arabinose consumed with an initial arabinose concentration of 10 g L^{-1}. This value is approximately 1.6X greater than the average amounts produced by S2 (51 mL H_2/ 20 mL culture), S1 (36 mL H_2/ 20 mL culture) or G (47 mL H_2/ 20 mL culture) inocula for the lowest concentration

of arabinose tested (10 g L^{-1}) (average of 44 mL H_2/20 mL culture or 2200 mL H_2/L culture). The amount of hydrogen produced from the three sludges in this study is similar to the amount of hydrogen produced in unacclimated sewage and distillery sludge (approximately 150 mL H_2/60 mL culture or 2500 mL H_2/L culture fed 20 g L^{-1} xylose (Lin et al., 2006). The rates of hydrogen production for initial concentration of 10 g L^{-1} of arabinose are higher in strain No. 2 (550 mL H_2/L culture-hr) compared to the three inocula used in this study (average of 2.6 mL H_2/20 mL culture-hr or 130 mL H_2/L culture-h) (Taguchi et al., 1994).

These differences in the amounts and rates of hydrogen may occur from non-optimal conditions within the batch reactors of the mixed cultures. For example, temperature and pH were shown to impact the rates and yields of hydrogen in strain No. 2 (Taguchi et al., 1994; Yang and Shen, 2006). In addition, micronutrient, macronutrient, and buffer concentrations can also influence the rates and yields of hydrogen production (Lin and Lay, 2004a; Lin and Lay, 2004b). Also, autoclaving to inhibit methanogenic activity may have depressed hydrogen producing activity compared to alternative methods such as bromoethanesulfonate (BES) or iodopropane (Zhu and Beland, 2006).

Volatile fatty acid (VFA) and ethanol production

Soluble fermentation products (SFP) released during fermentation are often used to evaluate the efficacy of hydrogen production. SFP for all batch reactors are shown in Table 3.2. For the S1 biomass, SFP increased to a maximum concentration of approximately 18 200 mg L^{-1} COD for 30 g L^{-1} arabinose and then decreased to a minimum concentration of approximately 2500 mg L^{-1} COD for 100 g L^{-1} arabinose. *n*-Butyrate was the most prevalent of the SFP for arabinose concentrations between 10 and 50 g L^{-1}. Valeric acid production was observed for concentrations between 30 and 75 g L^{-1} arabinose, ranging from 15 to 30% of the total SFP-COD. Production of valeric acid did not decrease the rate or the quantity of hydrogen as the highest amount of hydrogen production, rates, and shortest lag times were observed at initial arabinose concentrations of 30 and 40 g L^{-1} corresponding to valeric acid concentrations of 2200 and 2300 mg

L^{-1}, respectively. Previous studies have shown production of high concentrations of valeric acid with UASB reactors (Wang et al., 2007; Zhao et al., 2008). In addition, high valeric acid concentrations (approximately 2500 mg L^{-1}) were observed for acid pre-treated sludge batch reactors fed sucrose while batch reactors containing either heat treated (0 mg L^{-1}) or alkaline treated biomass (250 mg L^{-1}) produced little or none (Mu et al., 2007).

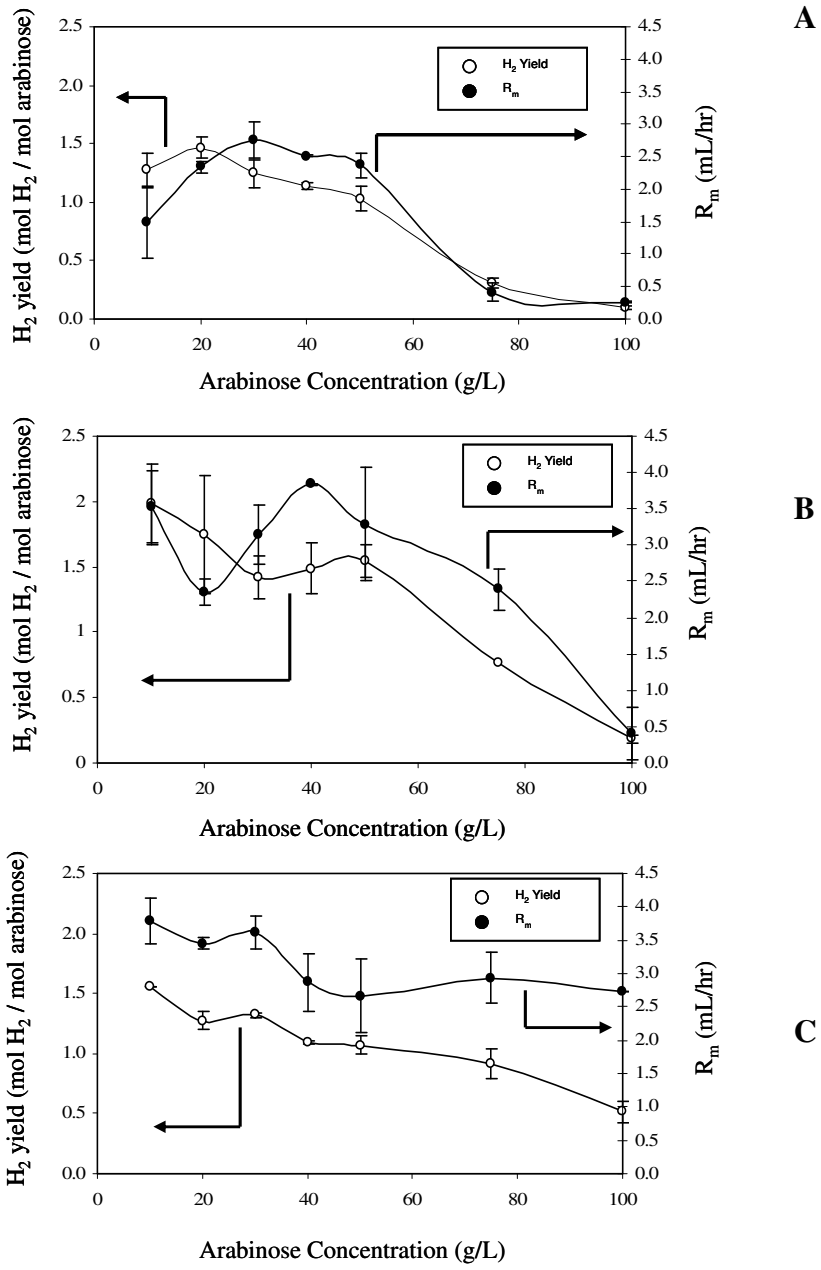

Figure 3.2. - Hydrogen yields and maximum hydrogen production rates (R_m) versus different arabinose concentrations for S1 (A), S2 (B), and G (C). Error bars represent one standard deviation of triplicate bottles.

Rates and yields of hydrogen production decreased in a sucrose-fed UASB reactor when concentrations of valerate increased above 275 mg L^{-1} (van Niel et al., 2004). In contrast, impact of valeric acid on biohydrogen production was inconclusive on this sucrose-fed UASB because hydrogen production rates and yields were at their highest and lowest at concentrations of valeric acid observed in the UASB reactor between approximately 450–500 mg L^{-1} (Lin and Lay, 2004a).

The total amount of SFP produced for S2 biomass was less than that produced for the S1. For arabinose concentrations between 10 and 50 g L^{-1}, the SFP produced were within 10% of each other, with the highest amount of SFP obtained for 40 g L^{-1} arabinose (approximately 10 000 mg L^{-1} COD). Similarly to the S1 biomass, the most-prominent of the VFA that were produced was n-butyrate, with relative amounts between 60–67% of the total SFP for arabinose concentrations ranging between 10 and 75 g L^{-1}. Unlike the S1 sludge, valeric acid was not detected for any arabinose concentrations.

For the granular biomass, the SFP production increased to a maximum concentration of approximately 12 000 mg L^{-1} COD. The total amount produced was generally higher when compared against the values for the S2 sludge but less than the amount produced for the S1 biomass. The largest percentage of n-butyrate occurred for 10 g L^{-1} arabinose and valeric acid was also produced. However, unlike the S1 inocula, production was observed for all concentrations of arabinose tested.

A COD balance for S1, S2, and G indicated that all of the metabolic products were identified (Table 3.2). Butyrate to acetate ratios (Bu/Ac) are often used to as an indicator of the extent of biohydrogen production. Previous studies indicate that efficient hydrogen production occurs for Bu/Ac ratios between 2.6 and 4.0 (Lin et al., 2006). Ranges of Bu/Ac varied between 0.0 for the S1 inoculum at 100 g L^{-1} and 5.1 for the granular inoculum at 40 g L^{-1} arabinose (Table 3.2). When the maximum hydrogen production was observed to be larger than 25 mL (or approximately 40% of the maximum) then the Bu/Ac ratio was between 3.2 and 5.1.

Table 3.2. - Production of soluble fermentation products (SFP) during fermentation with three different sludges under different initial substrate concentration

Arabinose ($g\ L^{-1}$)	Arabinose consumed (%)	SFP ($mgCOD\ L^{-1}$)	Formate (%)	Acetate (%)	Propionate + *i*-Butyrate (%)	*n*-Butyrate (%)	Valerate (%)	Ethanol (%)	COD balance (%)	Bu/Ac
Suspended Sludge(S1)										
10	100	10725	0.8	16.7	0.1	66.0	0.0	16.4	113.4	4.0
20	62.5	10567	0.8	16.4	0.8	64.4	0.0	17.6	96.2	3.9
30	56.9	18171	0.9	11.3	1.8	48.0	25.6	13.6	107.0	4.3
40	40.0	16684	1.2	11.5	1.9	46.7	27.0	12.9	103.8	4.1
50	32.8	14616	1.7	10.4	1.9	45.2	29.6	12.6	98.5	4.3
75	19.3	7721	4.5	47.8	14.8	15.4	14.6	5.6	109.0	0.3
100	11.1	2572	8.1	81.4	5.8	1.5	0.0	3.3	106.4	0.0
Suspended Sludge(S2)										
10	89.4	9269	1.3	15.5	8.0	59.5	0.0	16.1	111.1	3.8
20	53.4	9061	1.4	14.5	3.6	63.8	0.0	17.0	98.3	4.4
30	38.5	9913	1.2	14.3	0.1	66.7	0.0	17.7	98.0	4.7
40	31.7	9982	1.4	15.9	0.0	65.2	0.0	17.8	109.1	4.1
50	23.4	9521	2.2	14.2	5.1	61.9	0.0	16.9	115.6	4.4
75	18.2	6252	1.8	19.3	0.3	61.9	0.0	17.0	113.0	3.2
100	17.5	3386	3.8	25.6	5.3	5.8	0.0	60.4	100.2	0.2
Granular Sludge(G)										
10	100.0	7361	0.7	15.8	0.6	73.6	9.9	0.0	87.4	4.7
20	65.1	9174	1.3	11.8	0.8	54.3	18.3	14.2	93.9	4.6
30	51.1	10556	1.6	12.9	1.0	59.7	9.5	15.7	105.8	4.6
40	38.8	11167	1.8	10.7	1.4	55.0	17.5	14.5	108.2	5.1
50	33.5	10632	1.6	10.9	1.3	53.9	18.5	14.4	111.7	4.9
75	25.0	8853	1.8	15.5	1.6	62.0	3.2	16.7	110.1	4.0
100	18.0	5897	4.3	13.7	2.6	59.7	5.2	16.5	113.0	4.4

3.4 Conclusions

The potential of dark fermentation hydrogen production from arabinose by two suspended (S1, S2) and one granular (G) anaerobic sludge was assessed in batch assays and optimal hydrogen rates and yield were generally observed for concentrations between 10 and 40 g L^{-1} of substrate. Arabinose concentration of 100 g L^{-1} inhibited the hydrogen production although the granular sludge exhibited better hydrogen yields and production rates for concentrations between 50 and 100 mg L^{-1} than the suspended sludges. The largest amount of SFP produced for all three inocula was for n-butyrate. Also, valeric acid production was observed in some samples. In addition, hydrogen production increased when the Bu/Ac ratios were between 3.2 and 5.1.

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Inoculum type response to different pHs on biohydrogen production from arabinose a component of hemicellulosic biopolymers

Chapter 4

Abstract

Biohydrogen production from arabinose was examined using four different anaerobic sludges with different pHs ranging from 4.5 to 8.0. Arabinose (30 g L^{-1}) was used as the substrate for all experiments. Individual cumulative hydrogen production data were used to estimate the three parameters of the modified Gompertz equation. Higher hydrogen production potentials were observed for higher pH values for all the sludges. G2 (acclimated granular sludge) showed the highest hydrogen production potential and percentage of arabinose consumption compared to the other sludges tested. Granular sludges (G1 and G2) showed different behavior than the suspended sludges (S1 and S2). The differences were observed to be smaller lag phases, the percentage of acetate produced, the higher percentage of ethanol produced, and the amount of arabinose consumed. A high correlation (R^2 = 0.973) was observed between the percentage of n-butyrate and the percentage of ethanol in G1 sludge, suggesting that ethanol/butyrate fermentation was the dominant fermentative pathway followed by this sludge. In S1, however, the percentage of n-butyrate was highly correlated with the percentage of acetate (R^2 = 0.980). This study indicates that granular sludge can be used for larger pH ranges without reducing its capacity to consume arabinose and achieve higher hydrogen production potentials.

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4.1 Introduction

Hydrogen is now considered one of the alternatives to fossil fuels. It is preferred to biogas or methane because hydrogen is not chemically bound to carbon and therefore, combustion does not contribute to green house gases or acid rain (Nath and Das, 2004). While there are numerous ways to produce H_2 from renewable energy sources, currently the majority of H_2 is produced from fossil fuels (Das and Veziroglu, 2001). One alternative to sustainable H_2 energy production from renewable energy sources is through microbiological fermentation or photosynthesis. Dark fermentation produces H_2 at higher rates than photosynthesis and has the potential to combine organic waste management with simultaneous H_2 production (Levin et al., 2004).

Biological hydrogen production is affected by several environmental factors such as pH (Dabrock et al., 1992; Kondratieva and Gogotov, 1983). Fermentative hydrogen production occurs during the acidification stage and pH is one of important factors that affect this process. A change in system pH may result in decreased process efficiency. In general, the optimum initial pH for biohydrogen production is generally reported to be between 5.0 and 6.0 (Fang and Liu, 2002; Van Ginkel et al., 2001). However, there have been conflicted reports about the optimum pH value because the optimum pH in batch biohydrogen production was determined to be 9.0 with sucrose (Kim et al., 2004). There have been many studies examining the effect of pH in fermentative hydrogen production from glucose and sucrose using mixed microflora (Kawagoshi et al., 2005; Kim et al., 2004; Van Ginkel et al., 2001). Although the influence of pH on the fermentative biohydrogen production using arabinose one of the most common pentoses and a component of various biopolymers such as hemicellulose, is not well known. Previous studies reported biohydrogen production from arabinose using mixed cultures but the effect of pH is not described (Danko et al., 2008; Li et al., 2008). The effect of pH on the biohydrogen production from arabinose was examined using a pure culture but the range of pH values tested were limited and the soluble fermentation products were not identified (Taguchi et al., 1994). Understanding the effect of pH is necessary to develop arabinose-based

hydrogen fermentation applications, such as the use of agricultural wastes. The purpose of this study was to investigate the effect of initial pH on biohydrogen production from arabinose using mixed cultures in order to evaluate the feasibility of applying arabinose-based hydrogen fermentation in a continuous system.

4.2 Material and Methods

Batch experiments

Seed Sludges

Four different biomasses were tested for hydrogen production as follows: S1 (disperse anaerobic digester sludge from municipal WWTP), S2 (disperse anaerobic digester sludge from Municipal WWTP supplemented with fat), G1 (anaerobic granular sludge from Industrial WWTP from brewery waste) and G2 from a hydrogen producing reactor fed glucose and L-arabinose (1/1) 5g COD L^{-1} final concentration, during 120 d. S1, S2 and G1 sludges were heat treated to inhibit methanogenic activity.

Experimental procedures

The experiments were conducted using 125 mL serum bottles. L-arabinose was used as the substrate at an initial concentration of 30 gCOD L^{-1}. Four series of batch experiments were conducted, one for each biomass.

Anaerobic buffer (Colleran et al., 1992) (20 mL) was added to each vial containing 10gVSS L^{-1} of biomass and nutrients for bacterial growth (18 mL L^{-1} of macronutrients – $MgSO_4.7H_2O$: 30 g L^{-1}; KH_2PO_4: 28.3 g L^{-1}; NH_4Cl: 170 g L^{-1} and 1mL L^{-1} of micronutrients – $FeCl_2.6H_2O$: 2 g L^{-1}; H_3BO_3: 0.05 g L^{-1}; $ZnCl_2$: 0.05 g L^{-1}; $CuCl_2.2H_2O$: 0.038 g L^{-1}; $MnCl_2.4H_2O$: 0.5 g L^{-1}; $(NH_4)_6Mo_7O_{24}.4H_2O$: 0.05 g L^{-1}; $AlCl_3.6H_2O$: 0.09 g L^{-1}; $CoCl_2.6H_2O$: 2 g L^{-1}; $NiCl_2.6H_2O$: 0.092 g L^{-1}; $Na_2SeO_3.5H_2O$: 0.164 g L^{-1}; EDTA: 1 g L^{-1}; Resazurin: 0.2 g L^{-1}; HCl 37% (Zehnder et al., 1980).

Eight different pHs (4.5; 5.0; 5.5; 6.0; 6.5; 7.0; 7.5; and 8.0) were tested in triplicate. The initial pH of individual bottles was adjusted adding HCl or NaOH and flushing with 100% N_2, 20% CO_2 / 80% N_2 or 100% CO_2. The bottles were sealed, placed on a rotary shaker (150 rpm), and incubated at 37ºC. Hydrogen, VFA, and ethanol concentrations for the control inoculum (0 g L^{-1} of substrate) were subtracted from values obtained for each pH. Gas pressure was released using a glass syringe (20 and 50 mL capacity) by the Owen method (Owen et al., 1979). The amount of gas present in the headspace of each bottle was determined before and after releasing gas pressure.

Monitoring and analysis

Soluble COD was determined according to Standard Methods (APHA, 1989). Volatile fatty acids (VFA) (formate, acetate, propionate, iso-butyrate, n-butyrate, valerate), ethanol, and L-arabinose were determined by high performance liquid chromatography (Jasco, Japan) with a Chrompack column (6.5 x 30 mm^2). Sulfuric acid (0.01 N) was used as mobile phase at a flow rate of 0.7 mL min^{-1}. The column temperature was set at 60ºC. Detection of soluble products were made sequentially with an UV detector at 210 nm (VFAs) and a Refraction Index (RI) detector (ethanol and L-arabinose), respectively.

Hydrogen in the headspace of bottles was determined by gas chromatography (GC) using a pressure-lock syringe (0.2 mL injection volume) and a Hayesep Q column (80/100 mesh) and thermal conductivity detector (Varian 3300 Gas Chromoatograph) with nitrogen (30 mL min^{-1}) as the carrier gas. The injector, detector, and column temperatures were 120, 170, and 35 ºC, respectively. Methane was analysed by GC using a Porapack Q (100 -180 mesh) column with N_2 as the carrier gas (30 mL min^{-1}) and a thermal conductivity detector. The temperatures of the detector, injector, and oven were 110, 110 and 35 ºC, respectively.

The modified Gompertz equation was used to describe the progress of cumulative hydrogen production obtained from the batch experiments (Lay et

al., 1999; Zwietering et al., 1990). Using the cumulative hydrogen production data, corrected to STP conditions (0ºC and 1 atm), the maximum hydrogen production rates were estimated from the fit of the modified Gompertz equation (equation 1).

$$H(t) = P\exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where *H(t)* is cumulative hydrogen production (mL), *P* hydrogen production potential (ml), R_m maximum hydrogen production rate (mL h^{-1}), *e* = 2.71828…, λ lag-phase time (h), and, *t* time (h). R^2 values and the standard errors of each variable were calculated.

Principal Components Analysis (PCA)

PCA was used in order to find and interpret hidden complex relationships between features in a data set. PCA is a technique for summarizing the information contained in variables by a few weighted components as a mean of reducing the number of variables needed in an analysis. Correlating features were converted to the so-called factors which are themselves noncorrelated (Einax et al., 1997). PCA modeling shows the correlation structure of data matrix *X*, approximating it by a first term $1 * \overline{X}'$ representing the variables average plus a matrix product of lower dimension (*TP'*), called the principal components, plus a matrix of residuals (*E*).

$$X = 1 * \overline{X}' + TP' + E \quad (2)$$

SIMCA-P (Umetrics AB) software package was used to perform the PCA; it iteratively computes one principal component at a time, comprising a score

vector *t* and a loading vector *p*. The score vectors contain information on how the samples relate to each other (matrix *T*). Otherwise, the loading vectors define the reduced dimension space and contain information on how the variables relate to each other (matrix *P*). Usually, a few PC (2 or 3) can express most of the variability in the database when a high degree of correlation among data exists.

The criterion used to determine the model dimensionality (number of significant components) is cross validation (CV). Part of data is kept out of the model development, and then is predicted by the model and compared with the actual values. The prediction error sum of squares (PRESS) is the squared differences between observed and predicted values for the data kept out of the model fitting. This procedure is repeated several times until the data elements have been kept out once and only once. Therefore, the final PRESS has contributions from all data. For every dimension, SIMCA computes the overall PRESS/SS, where SS is the residual sum of squares of the previous dimension. A component is considered significant if PRESS/SS is statistically smaller than 1.0.

Partial Least Squares regression (PLS)

PLS is an iterative algorithm that extracts linear combinations of the essential features of the original data *X* while modeling the *Y* data dependence on the data set, being well suited for multivariate calibration. The most important advantage of this method reports to the non-problematic handling of multicollinearities relying on an iterative algorithm, which makes possible the treatment of data with more features than objects (Einax et al., 1997).

In this method, the latent variables *u* (matrix *U*) are used for modeling the objects separately in the matrix of *Y* dependent data, whereas, the *t* variables (matrix *T*) are used for modeling the objects separately in the *X* matrix of independent data. The latent variables *U* and *T* are the basis of the regression model and are determined by:

$$U = AT + E \qquad (3)$$

PLS components matrix *A* and error matrix *E*, in an iterative process with the centred matrices of *X* and *Y* as starting points (Einax et al., 1997).

SIMCA-P (Umetrics AB) software package was used to perform PLS analysis from the data set. This software iteratively computes one PLS at a time, that is, one vector for each of X-scores (*t*), Y-scores (*u*), weights (*w*) expressing the correlation between *X* and *U*, weights (*c*) expressing the correlation between *Y* and *T* and loadings (*p*). The PLS components are calculated in descending order of importance. For the response variables (*m*) in *Y*, the multiple correlation coefficient (R^2Y_{cum}) or goodness of fit is given by:

$$R^2Y_{cum} = \sum R^2Y_a \qquad (4)$$

where, R_2Y_a is the sum of squares of the entire Y's explained by each extracted component (*a*).

4.3 Results and Discussion

Effect of pH on hydrogen production potentials, rates and lag times

Biohydrogen production from arabinose was examined using initial pH values ranging from 4.5 to 8.0 for four different anaerobic sludges. The initial substrate concentration was 30 g L^{-1} COD for all experiments with 0 g L^{-1} serving as control. Individual cumulative hydrogen production data were used to estimate the three parameters of the modified Gompertz equation (maximum hydrogen production rate, hydrogen production potential, and duration of the lag phase). Hydrogen production occurred for all four sludges but there were differences in the yields, lag times, and rates (Table 4.1). Methane production was not detected in any of the batch cultures indicating that methanogenic activity was inhibited. pH was measured at the end of each batch experiment and the values were determined to be approximately 5.0 for all the biomasses tested (data not shown).

G1 was determined to have the highest hydrogen production potential (61.6±0.1mL) at pH of 6.5 while the highest hydrogen production rate (2.3±0.2 mL h^{-1}) was obtained at a pH of 7.0. Also, the shortest lag time (10.6±2.4 h) was detected at a pH of 8.0 (Table 4.1). G2 was determined to have the highest hydrogen production potential (137.2±9.6 mL at pH 7.5) when compared with the other sludges tested. G2 showed the highest hydrogen production rate (2.9±0.2 mL h-1) at pH 7.5 and lower lag phase 11±1.8 h at pH 7.0). Concerning the S1 sludge, the highest hydrogen production potential (51.1±1.3 mL) and rate (2.8±0.4 mL h-1) occurred at a pH of 7.0. The shortest lag time was obtained with pH 6.0 (Table 4.1).For S2 sludge, the highest hydrogen production potential was observed with pH 8.0 (58.1±1.8 mL) and the maximum rate (4.8±1.4 mL h^{-1}) was obtained with pH 7.5.

Higher hydrogen production potentials corresponding to higher pH values have been observed in other studies (Kim et al., 2004). When comparing all four sludges, G2 obtained the highest hydrogen production potential (137.2 mL) and S2 obtained the largest hydrogen production rate (4.8 mL h^{-1}) at a pH of 7.5, while G1 obtained the shortest lag time (10.6 h) at a pH of 8.0. Comparing these results with previous studies using mixed cultures (Li et al., 2008) a higher hydrogen production potential and hydrogen production rates were obtained as well as a significant reduction in lag phases. Li (2008) reported a cumulative hydrogen yield of 34mL, hydrogen production rate of 0.8mL h-1 and a lag phase of 68h using a pH of 6.

Effect of pH on arabinose consumption and hydrogen yields

Hydrogen yields were calculated for all batch reactors based on the amount of arabinose consumed and the amount of hydrogen produced. The results are shown in (Table 4.1).

Table 4.1. - Modified Gompertz equation parameter values, percentage of arabinose consumed, COD balance, hydrogen yields for the different pH's tested

G1 sludge							
pH	P (mL)	Rm (mL/h)	lambda (λ)	R^2	arabinose consumed (%)	COD Balance (%)	Yield ($molH_2$/mol arab consumed)
4.5	27.7± 0.6	0.7± 0.1	21.9± 2.3	0.99	42.1	105.3	0.8± 0.1
5.0	26.2± 0.5	0.9± 0.1	17.1± 1.2	0.99	41.1	107.4	0.8± 0.2
5.5	32.2± 0.7	0.9± 0.1	15.9± 1.9	0.99	44.8	115.3	0.8± 0.1
6.0	54.3± 1.5	1.2± 0.1	17.2± 1.9	0.99	50.5	99.6	1.2
6.5	61.6±0.1	2.1± 0.1	15.2± 0.9	1.00	53.8	99.5	1.3
7.0	56.4± 1.2	2.3± 0.2	12.3± 1.2	0.99	52.0	100.6	1.2± 0.1
7.5	51.2± 1.1	2.0± 0.2	11.6± 1.4	0.99	52.6	100.3	1.1± 0.1
8.0	41.3± 1.7	1.8± 0.4	10.6± 2.4	0.96	54.6	93.3	0.9± 0.1

G2 Sludge							
pH	P (mL)	Rm (mL/h)	lambda (λ)	R^2	arabinose consumed (%)	COD Balance (%)	Yield ($molH_2$/mol arab consumed)
4.5	0.0	0.0	0.0	na	19.9	90.2	0.0
5.0	11.8± 0.1	0.9±0.1	50.2±0.7	0.99	24.8	87.0	0.5± 0.18
5.5	47.2±0.4	2.1±0.1	19.3±0.6	0.99	50.0	93.7	1.1± 0.1
6.0	93.0±0.4	2.6±0.04	18.8±0.3	0.99	72.3	109.8	1.5± 0.2
6.5	111.8±1.4	2.9±0.16	14.5±1.1	0.99	80.7	116.4	1.5 ± 0.03
7.0	97.4±1.9	2.3±0.2	11.0±1.8	0.99	75.7	117.0	1.4 ± 0.21
7.5	137.2±9.6	1.7±0.2	13.7±4.7	0.97	92.4	115.8	1.5 ± 0.05
8.0	136.7±6.4	1.9±0.2	15.4±3.3	0.98	97.6	114.5	1.5 ± 0.05

S1 Sludge							
pH	P (mL)	Rm (mL/h)	lambda (λ)	R^2	arabinose consumed (%)	COD Balance (%)	Yield ($molH_2$/mol arab consumed)
4.5	0.4± 0.04	1.0± 0.2	80.0± 0.2	0.89	8.8	104.8	0.1
5.0	11.3± 0.1	0.8± 0.04	56.1± 0.4	1.00	13.2	113.0	0.7± 0.1
5.5	19.3± 0.8	1.0± 0.2	25.9± 1.7	0.98	11.3	114.0	2.0± 0.6
6.0	24.4± 0.5	1.8±0.2	18.7± 0.9	0.99	22.2	119.8	1.3± 0.3
6.5	38.0± 1.1	1.9± 0.3	29.5±1.7	0.99	28.8	107.7	1.6± 0.1
7.0	51.1± 1.3	2.8± 0.4	24.1± 1.3	0.99	29.6	108.9	2.0± 0.1
7.5	47.4± 1.8	2.5± 0.5	22.6±2.0	0.98	33.3	106.1	1.7
8.0	46.9± 1.2	2.2± 0.3	24.0± 1.4	0.99	28.5	106.5	2.0± 0.3

S2 Sludge							
pH	P (mL)	Rm (mL/h)	lambda (λ)	R^2	arabinose consumed (%)	COD Balance (%)	Yield ($molH_2$/mol arab consumed)
4.5	23.2 ± 1.3	0.5± 0.1	26.0± 2.9	0.98	19.9	95.2	1.3 ± 0.1
5.0	35.7± 2.1	1.2± 0.2	37.5± 2.8	0.98	24.9	95.1	1.5 ± 0.1
5.5	34.5± 1.5	1.4± 0.2	31.9± 2.1	0.99	23.0	101.9	1.7± 0.2
6.0	49.4± 0.5	1.9± 0.1	28.3± 0.6	1.00	22.1	101.3	2.5
6.5	54.4± 0.4	3.3±0.1	27.9±0.4	1.00	34.5	102.9	1.8 ± 0.2
7.0	47.1±0.5	3.0±0.4	19.4±1.5	1.00	32.4	99.1	1.7 ± 0.1
7.5	56.3±1.5	4.8±1.4	32.8±2.4	1.00	39.6	108.9	1.2 ± 0.7
8.0	58.1±1.8	2.4±0.3	28.2±1.6	1.00	39.7	103.0	1.7

The highest hydrogen yield was obtained with S2 (2.5mol H_2/mol arabinose consumed) at pH 6.5. The highest hydrogen yield obtained for S1 was 2.0 mol/H_2 mol arabinose consumed with a pH of 7.0 and 8.0 and the highest hydrogen yield obtained for G2 was 1.5 mol/H_2 mol with pH values of 6.0, 6.5, 7.5 and 8.0. G1 had the smallest hydrogen yield (1.3 mol/H_2 mol arabinose consumed; pH = 6.5) when compared with the other biomasses. However, the minimum amount of arabinose consumed for G1 was at least 41% for all pH values. For S2, the highest percentage of arabinose consumed was 39.7% at pH 8.0. The highest percentage for S1 was only 33.3% at a pH of 7.5. G2 was observed to have the highest percentage of arabinose consumption (97%) at a pH of 8.0.

The yields obtained in this study are less than the theoretical value (3.3 mol H_2/mol arabinose). Although, compared to the values obtained in a previous study that used xylose (pentose) (20gCOD L^{-1}) as a substrate (Lin and Cheng, 2006) the maximum yields obtained in this study are slightly higher. The highest yield obtained in the previous study using xylose (2.25 mol H_2/mol xylose) was observed at a pH of 6.5, while in the present study we were able to obtain 2.5 mol H_2/mol arabinose consumed using S2 at pH 6.0. The yields obtained in the present study were significantly higher than those obtained in a previous study that also used arabinose as the substrate (10g L^{-1}) and mixed culture for hydrogen production (9.7mL H_2/g arabinose consumed corresponds to 0.05 mol H_2/mol arabinose consumed) (Li et al., 2008). The yields and amounts for hydrogen production were also different for this study when compared against the pure culture of *Clostridium* (strain No.2) fed with arabinose (10g L^{-1}) (Taguchi et al., 1994). The maximum yield for the strain No. 2 (2.2 mol H_2/mol arabinose consumed) was similar to S1 and S2 but was higher than the yields obtained with G1 and G2. The maximum amount of hydrogen production from *Clostridium* (strain No.2) with controlled pH was 3600 mL H_2 L^{-1}culture and with uncontrolled pH was 2000 mL H_2 L^{-1}culture (Taguchi et al., 1994). These values are similar to the maximum amounts of hydrogen production from S1 (2550 mL H_2 L^{-1}culture), S2 (2900 mL H_2 L^{-1} culture), and G1 (3100 mL H_2 L^{-1} culture). However, G2 produced almost twice as much hydrogen (6850 mL H_2 L^{-1} culture) as strain No. 2.

Effect of pH on VFAs and ethanol production

Soluble fermentation products (SFP) released during fermentation are often used to evaluate the efficiency of hydrogen production. The percentage of each VFA and ethanol at the end of each batch test for each pH tested is shown in Table 4.2.

For G1 sludge, the SFP production achieved a maximum concentration of 19144 mg COD L^{-1} at pH 5.5. All other pH values produced approximately 15000 mg COD L^{-1}. The total amount of SFP produced was higher when compared against the values obtained with S1 and S2. G2 obtained the highest SFP production compared to the other sludges (29717 mg COD L^{-1} at pH 8.0. In addition, SFP was higher than 22931 mg COD L^{-1} when pH values were higher than 5.5. The highest percentage of ethanol for all pH values was observed for G1 and G2 (Table 4.2). n-Butyrate was the second most abundant SFP fort all pH values. Acetate was produced but corresponded to less than 6%. The presence of large amounts of ethanol and small amounts of acetate may be one of the reasons for the smaller hydrogen yields obtained with G1 and G2 even though higher percentages of arabinose consumption were observed. This suggests that the system was following an ethanol type fermentation (Thauer et al., 1977; Moat, 1979).

Regarding the S1 sludge, the SFP production achieved a maximum concentration of 10779 mg COD L^{-1} with a pH of 7.5. The most prominent SFP present for pH values greater than 4.5 was n-butyrate, corresponding to values between 50 and 65% of the SFP produced, followed by acetate (approximately 20%) and ethanol (approximately 16%) (Table 4.2). This suggests that the hydrogen is being produced via butyrate-acetate fermentation (Noike and Mizuno, 2000). Acetate had the highest percentage of SFP production (approximately 70%) at a pH of 4.5. However, the amount of arabinose consumed was very low (8.8%). S2 produced similar amounts of SFP to S1 although the distribution was slightly different.

Table 4.2. - Total COD from VFA's and ethanol and percentage of each soluble fermentation products (SFP) at the end of each batch test, for the different pHs

G1 Sludge								
pH	VFAs+Ethanol (mgCOD/L)	Percentage (%)						
		Formate	Acetate	Prop	i-but	n-but	valer	ethanol
4.5	15257	0.0	3.4	0.0	0.0	13.8	0.0	82.8
5.0	15710	0.0	3.8	0.0	0.0	14.4	0.0	81.8
5.5	19144	0.1	8.4	0.0	0.0	28.6	0.0	62.9
6.0	15050	0.1	4.7	0.0	0.0	23.4	0.0	71.8
6.5	15765	0.3	6.1	0.0	0.0	29.6	0.0	63.9
7.0	15734	0.8	4.9	0.0	0.0	27.7	0.0	66.5
7.5	15945	0.6	6.2	0.0	0.0	28.4	0.0	64.8
8.0	14643	0.6	3.7	0.0	0.0	34.4	0.0	61.2

G2 Sludge								
pH	VFAs+Ethanol (mgCOD/L)	Percentage (%)						
		Formate	Acetate	Prop	i-but	n-but	valer	ethanol
4.5	574	0.0	0.0	0.0	0.0	17.8	0.0	82.2
5.0	2862	0.0	10.5	3.1	0.0	22.0	0.0	64.5
5.5	12278	0.0	9.9	3.8	0.0	30.4	0.0	56.0
6.0	22931	0.0	10.1	1.4	0.0	31.2	0.0	57.3
6.5	27274	0.0	9.9	1.2	0.0	31.2	0.0	57.7
7.0	26276	0.0	8.2	1.5	0.0	32.9	0.0	57.4
7.5	29400	0.0	8.2	0.9	0.0	31.1	0.0	59.8
8.0	29717	0.0	8.3	0.7	0.0	29.8	0.0	61.2

S1 Sludge								
pH	VFAs+Ethanol (mgCOD/L)	Percentage (%)						
		Formate	Acetate	Prop	i-but	n-but	valer	ethanol
4.5	993	7.9	64.1	8.3	4.3	6.4	0.0	0.0
5.0	3976	1.6	24.1	3.7	0.9	48.8	0.0	19.7
5.5	5547	1.1	20.9	1.8	0.0	60.8	0.0	15.4
6.0	8119	1.0	17.6	1.6	0.6	64.8	0.0	15.7
6.5	10224	1.7	23.3	0.0	0.4	57.0	0.2	16.2
7.0	10410	0.9	19.7	0.0	0.0	62.3	0.2	16.2
7.5	10779	1.1	19.7	0.0	0.0	61.5	0.2	17.3
8.0	10340	1.2	17.9	0.0	0.0	62.2	0.4	17.1

S2 Sludge								
pH	VFAs+Ethanol (mgCOD/L)	Percentage (%)						
		Formate	Acetate	Prop	i-but	n-but	valer	ethanol
4.5	4001	0.2	20.5	0.0	0.0	71.0	0.0	8.3
5.0	5168	0.2	23.4	0.0	0.0	70.5	0.0	5.8
5.5	5942	0.4	24.4	0.0	0.0	74.6	0.0	0.6
6.0	5433	0.9	33.5	0.0	0.0	61.0	0.0	4.3
6.5	9974	1.4	25.4	0.0	0.0	72.3	0.0	0.7
7.0	8271	0.9	23.1	0.0	0.0	74.8	0.0	0.4
7.5	11097	1.8	24.1	1.5	0.3	54.9	0.0	16.9
8.0	11465	0.7	22.2	0.0	0.3	60.0	0.0	16.5

The highest amount of SFP (11465 mgCOD L^{-1}) was observed at a pH of 8.0 (Table 4.2). The most prominent SFP present was n-butyrate, corresponding to approximately 70% of the total SFP produced at pH values of 4.5, 5.0, 5.5, 6.5 and 7.0, approximately 60% with pH values of 6.0 and 8.0, and 55% with a pH of 7.5 (Table 4.2). Acetate was the second most abundant VFA for all pH values (approximately 20%), except at pH 6.0 (approximately 34%). This pH value (6.0) corresponded to the highest hydrogen yield (2.5 molH_2/mol arabinose consumed) and the highest percentage of acetate in all experiments, after pH 4.5 from S1 sludge. Ethanol was present in all samples corresponding to less than 10% of the SFP for all pH values except for 7.5 and 8.0. This suggests that the hydrogen is being produced via butyrate-acetate fermentation (Noike and Mizuno, 2000). For all batch tests the COD balance indicated that the major metabolic products were identified (Table 4.1).

Principal Components Analysis (PCA)

Principal Components Analysis (PCA) was performed to visualize the main differences between the 4 biomasses tested. The dataset consisted of 13 variables and 32 samples. All variables were autoscaled to unit variance, avoiding that some variables would be more important than others because of scale effects. The 3 first Principal Components (PC) contained 82.4% of the total variability present in the dataset. The use of more components did not significantly improve the robustness of the model. The plane t[1] vs. t[2] (Figure 4.1a) shows that the granular sludges (G1 and G2) presented different behaviour than the suspended biomasses (S1 and S2). The score (t_i) of an observation (i) on a principal component PC_j (t_iPC_j) is the weighted sum of the original variables (x_i). The weights (p_i) are called the loadings of the variables on that *PCj*. The loading of a variable is related to its variation (Massart and Heyden, 2005).

$$t_i\,(PC_j) = \sum [p_i\,(PC_j) * x_i] \qquad (6)$$

Therefore, analyzing Figure 4.1b (p[1] *vs.* p[2]) we verify that the differences of granular sludges comparing to suspended sludges are explained by smaller concentrations of VFA's, hydrogen yield (YH2) and percentages of acetate and n-butyrate, and also by higher % of ethanol and arabinose consumed.

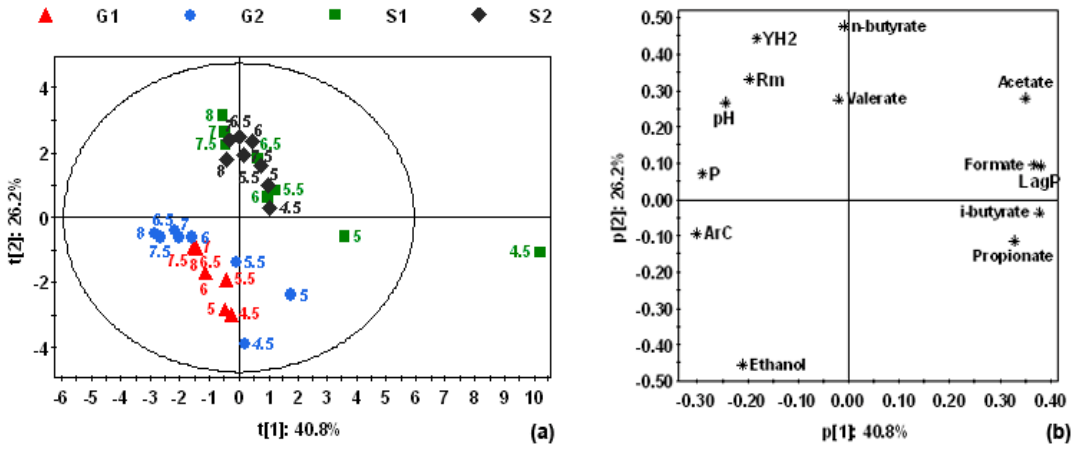

Figure 4.1. - Score map (a) and Loading map (b) obtained with Principal Component Analysis for all assays.

The sample corresponding to a pH of 4.5 from S1 (Figure 4.1a) is an outlier of the model because it shows higher percentages of formate, propionate, i-butyrate, and acetate, and smaller percentages of ethanol and arabinose consumed, with large lag phases, and small H_2 production potentials (P).

Partial Least Squares (PLS)

In order to determine the relationship between parameters, a Partial Least Squares (PLS) regression was performed, individually, to each of the biomasses datasets with P (Hydrogen production potential) as the Y variable, and Lag-Phase time, R_m (maximum hydrogen production rate), Arabinose Consumed, Volatile Fatty Acids, and Ethanol as the X variables.

When the PLS regression was performed no significant improvement in the prediction ability occurred for more than two latent variables in the P study attaining a value for the multiple correlation coefficient (goodness of fit) of 97.8, 94.2, 98.9 %, and 96.2, respectively for the S1, S2, G1, and G2 sludge datasets (Figure 4.2).

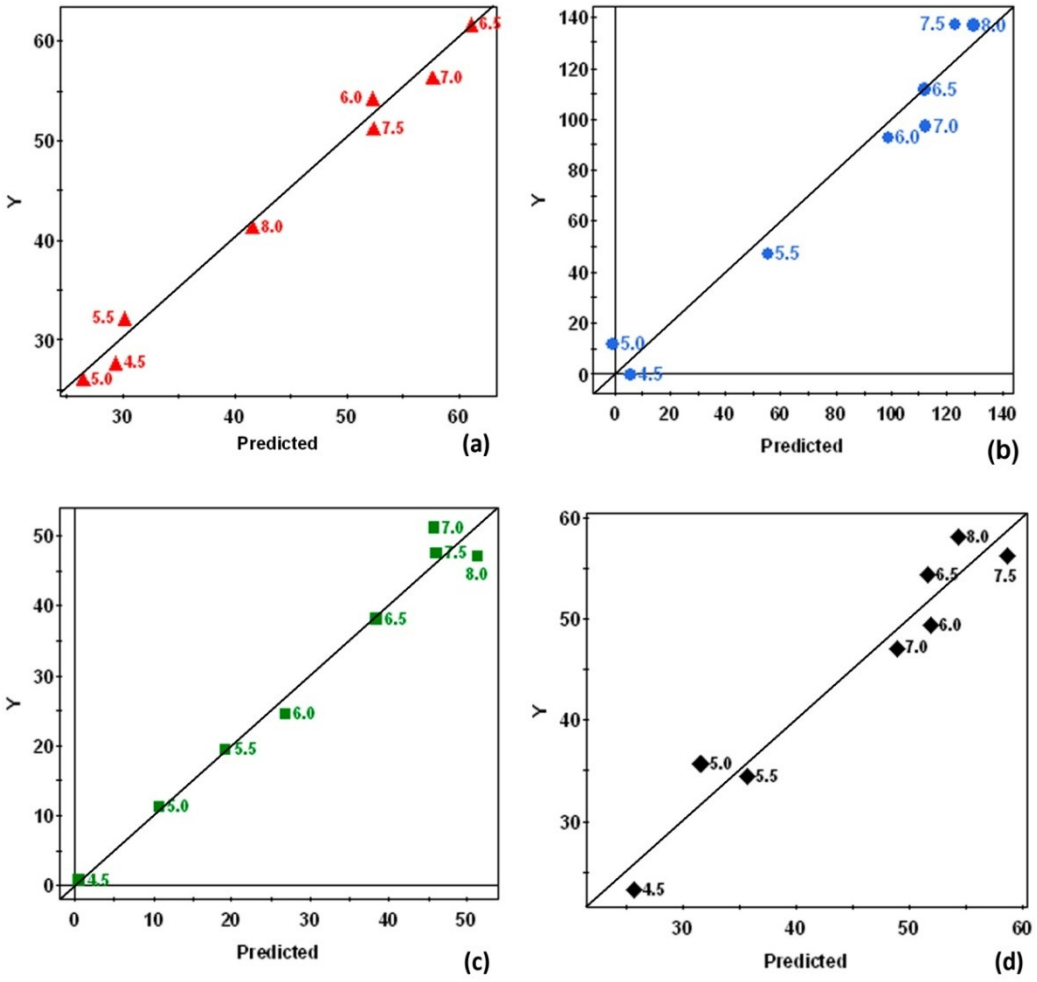

Figure 4.2. - Hydrogen Potential Production (P), observed and predicted, with two latent variables for: (a) G1; (b) G2; (c) S1; and (d) S2.

The loading plots *w*c* display both the correlation between the *X*-weights (*w**) and *Y*-weights (*c*), and thereby the correlation structure between *X* and *Y*. One sees how the *X* and *Y* variables combine in the projections, and how the *X* variables relate to *Y* and to each other. These weights are selected so as to maximize the covariance between *T* and *U*, thereby indirectly *T* and *Y*. It is

important to note that variables with equivalent (positive or negative) weights are highly correlated. The variables with similar weights (*w*c*) are directly correlated, and variables are inversely proportional if their weights are symmetric, *i.e.* situated in opposite quadrants of the graph.

A high correlation (R^2 = 0.973) was observed between the percentage of n-butyrate and the percentage of ethanol for G1 sludge (Figure 4.3a). This suggested that the fermentation is following the butyrate/ethanol pathway corresponding to the lower yields of hydrogen obtained.

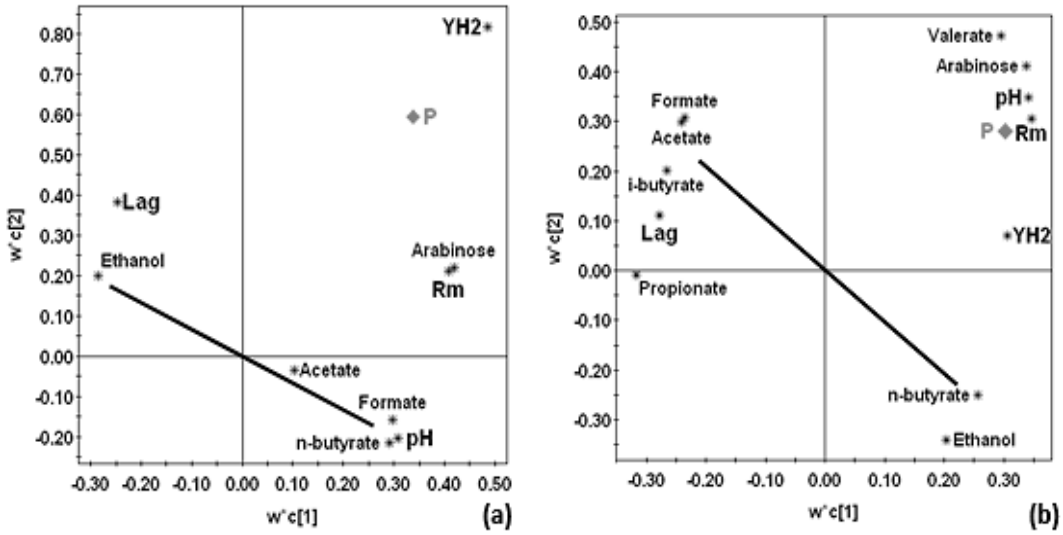

Figure 4.3. - Loading Maps for G (a), S1 (b), with P as Y variable.

It is shown in Figure 4.3b, that the percentage of n-butyrate is highly correlated with the percentage of acetate (R^2 = 0.980) for the S1 sludge. This suggests that the system is following butyrate-acetate type fermentation with butyrate in excess.

Acclimated granular sludge

G2 sludge was obtained from hydrogen producing continuous system and the batch experiments revealed that this biomass achieved higher hydrogen production potentials and a higher percentage of arabinose consumption with a

very large range of pHs (Table 4.1). This suggests that biomass acclimatization is very important to achieve higher hydrogen production values and higher percentages of substrate consumption. For a continuous system, high hydrogen production rates and small lag phases as well as tolerance to pH variations are essential. Suspended sludges showed higher yields of hydrogen production when compared to the granular sludges but were observed to have lower hydrogen production potentials and percentages of arabinose consumption and also longer lag phases. In general, granular sludges showed the highest hydrogen production potentials within a larger range of pH values that demonstrated a higher tolerance to pH changes. On the other hand, the maintenance of high biomass concentrations inside the reactors, such as those observed in granule based systems, is necessary for a stable hydrogen production.

4.4 Conclusions

In the present study, all the sludges tested showed higher hydrogen production potentials values with the utilization of higher initial pH values. Granular sludges obtained smaller lag phases and higher percentages of arabinose consumption. G2 (acclimatized granular sludge) showed highest hydrogen production potential values and percentage of arabinose consumption. Granular sludges (G1 and G2) showed different behavior than the suspended sludges (S1 and S2). The differences were observed to be smaller lag phases, the percentage of acetate produced, the higher percentage of ethanol produced, and the amount of arabinose consumed. The percentage of n-butyrate is highly correlated with the percentage of acetate (R^2 = 0.980) for S1 suggesting an acetate/butyrate main pathway. High correlation (R^2 = 0.973) was also observed between the percentage of n-butyrate and the percentage of ethanol for G1. This suggested that the fermentation is following the butyrate/ethanol pathways which corresponded to the lower yields of hydrogen obtained. This study suggests that acclimatization of biomass is very important to achieve higher hydrogen production potentials and substrate consumption. Granular sludge can be used

for larger pH ranges without losing its hydrogen production potential and arabinose uptake capacity when compared with suspended sludges.

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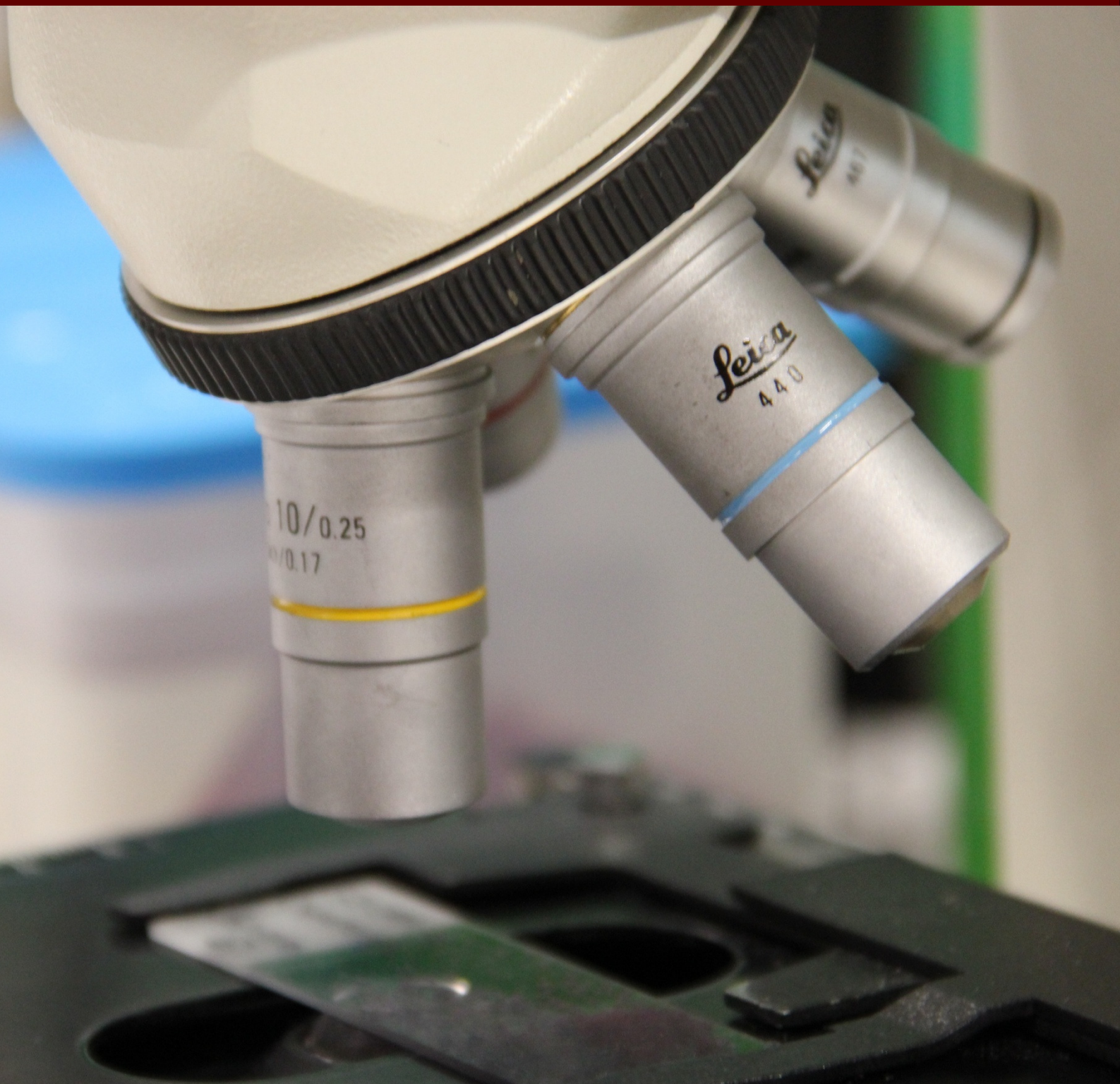
Strategies for suppress hydrogen-consuming microorganisms affect macro and micro scale structure and microbiology of granular sludge

Chapter 5



Abstract

Treatment of anaerobic granules with heat and two chemical treatments, i.e. contacting with 2-bromoethanesulfonate (BES) and with BES+Chloroform, were applied to suppress the activity of hydrogen-consuming microorganisms. Three mesophilic expanded granular sludge bed (EGSB) reactors – R_{Heat}, R_{BES} and $R_{BES+Chlo}$ – were inoculated with the respective treated sludges and fed with synthetic sugar-based wastewater (5 $gCODL^{-1}$, HRT 20-12h). Morphological integrity of granules and bacterial communities were assessed by quantitative image analysis and 16S rRNA gene based techniques, respectively. Hydrogen production in R_{Heat} was under 300 $mLH_2L^{-1}d^{-1}$, with a transient peak of 1000 $mLH_2L^{-1}d^{-1}$ after decreasing HRT. In $R_{BES+Chlo}$ hydrogen production rate did not exceed 300 $mLH_2L^{-1}d^{-1}$ and there was granule fragmentation, release of free filaments from aggregates, and decrease of granule density. In R_{BES}, there was an initial period with unstable hydrogen production, but a pulse of BES triggered its production rate to 700 ± 200 $mLH_2L^{-1}d^{-1}$. This strategy did not affect significantly granules structure. Bacteria branching within *Clostridiaceae* and *Ruminococcaceae* were present in this sludge.

This work demonstrates that methods applied to suppress the activity of hydrogen-consuming microorganisms can cause changes in the macro- and microstructure of granular sludge, which can be incompatible with operation of high-rate reactors.

Biotechnology and Bioengineering (2011), 108 (8), 1766-1775

5.1 Introduction

Hydrogen can be continuously generated from renewable organic materials in mixed culture dark fermentation processes. These processes offer several advantages over pure culture fermentations, namely no need for media sterilization, robustness and increased adaptation capacity offered by the microbial consortia, possibility of mixed substrates co-fermentation, and better suitability for continuous processing (Kleerebezem and van Loosdrecht, 2007; Temudo et al., 2007). However, in anaerobic mixed culture systems, hydrogen produced by fermentative bacteria such as *Clostridium* or *Enterobacter* is often readily consumed by hydrogenotrophic methanogens or homoacetogenic bacteria (Lovley and Klug, 1983), or is used as electron donor by sulphate- and nitrate-reducing bacteria. Therefore, activity of these different types of H_2-utilizing microorganisms must be inhibited, while preserving activity of H_2-producing bacteria. For this, seed sludge of hydrogen-producing anaerobic reactors is often pre-treated with heat, electric current, air, or chemicals (e.g. bases, acids or specific inhibitors). Heat treatment has been commonly used for inactivating methanogenic archaea; this method allows also a screening for H_2-producing bacteria, as many of these mesophilic bacteria are spore-formers (Lay et al., 1999). However, it has been reported that heat treatment was unable to completely inhibit H_2-consuming microorganisms (Oh et al., 2003). Addition of chemicals, such as 2-bromoethanesulfonate (BES), an analogue of coenzyme M in methanogens and an inhibitor of methane-producing archaea (Dimarco et al., 1990; Sparling et al., 1997), and chloroform, thought to inhibit methanogenesis and homoacetogenesis (Chidthaisong and Conrad, 2000), may aid in the overall inhibition of H_2-utilizers.

High-rate anaerobic reactors, such as the expanded granular sludge bed (EGSB) reactor, can be adapted for hydrogen production. However, microbial selection and development of H_2-producing granules from suspended sludge inocula, requires a long start-up time (Mu and Yu, 2006; Yu and Mu, 2006; Zhang et al., 2007). Using environmental pressure for promoting the development of H_2-producing microorganisms already present in matured anaerobic granules can be

used as a strategy for decreasing start-up times of high-rate H_2-producing reactors as it eliminates the need for a granulation period. Nevertheless, methods to inhibit H_2-consuming microorganisms may have an effect on the structural and morphological properties of granular sludge. Morphological parameters of H_2-producing granules have been assessed only in few studies (Mu and Yu, 2006; Zhang et al., 2008a), and the effect of heat or chemical treatments on granules structure and integrity was never studied.

Assessment of aggregates shape and size descriptors, such as free filaments length in the bulk, density of granules, and diameter of micro- or macro-aggregates, which have been suggested as suitable to quantify phenomena of granules erosion, fragmentation and washout (Araya-Kroff et al., 2004, Amaral et al., 2004, Abreu et al., 2007, Costa et al., 2009), can give more insights on the morphological changes deriving from heat or chemical treatment of matured anaerobic granules.

In the present work, changes in the macro- and micro-scale structural morphology and in the microbial composition of granules subjected to pretreatment with heat, BES and BES+Chloroform were studied. A comprehensive link between morphology and microbial community structure could be established.

5.2 Material and Methods

Source of sludge sample and sludge treatments

Anaerobic granular sludge was obtained from an industrial WWTP from brewery wastewater. Volatile suspended solids (VSS) content of the sludge was 48 mgVSS g^{-1}. This sludge was submitted to different treatments in order to inhibit the activity of hydrogen-consuming microorganisms, as follows:

Heat treatment. Granular sludge was boiled at 100ºC for 15 min (Lay et al., 1999). Sludge S_{HEAT} was used as inoculum in reactor R_{Heat}.

BES treatment. Granular sludge was incubated for 72 h at 37ºC with 15 mM 2-bromoethanesulfonate (BES). Sludge S_{BES} was used as inoculum in reactor R_{BES}.

BES+Chloroform treatment. Anaerobic granular sludge was incubated for 72h at 37ºC with 15 mM BES plus 30 µM chloroform. Sludge $S_{BES+Chlo}$ was used as inoculum in reactor $R_{BES+Chlo}$.

Experimental setup

The experiments were carried out in three EGSB reactors with an overall height of 1.95 m and an internal diameter of 21 mm. Total volume was 1.30 L and working volume 0.7 L. Reactors R_{HEAT}, R_{BES} and $R_{BES+Chlo}$ were inoculated with 400 mL of differently treated anaerobic granular sludge, S_{HEAT}, S_{BES} and $S_{BES+Chlo}$, respectively (treatments described above). Reactors were operated continuously and fed with a mixture of glucose (13mM) and L-arabinose (16mM) with a total chemical oxygen demand (COD) concentration of 5g L^{-1}. Macronutrients, necessary for cell growth, were added to the feed (0.6 mL g^{-1}COD of a stock solution containing 30 g L^{-1} $MgSO_4.7H_2O$, 28.3 g L^{-1} KH_2PO_4, and 170 g L^{-1} NH_4Cl). Sodium bicarbonate was added as alkalinity source (1 to 2 g L^{-1}) and pH was maintained around 5.5. Temperature of the reactors was kept at 37 ± 1 ºC by means of an external jacket for water circulation. Reactors were operated with a hydraulic retention time (HRT) of 20h during the first 40 days; afterwards HRT was decreased to 12h. Superficial velocity was set at 8.0 m h^{-1} by using an internal liquid recirculation. A pulse of BES (15mM) was added to R_{BES} on day 57 of operation. Pulses of BES and chloroform (15mM and 30µM respectively) were also added to $R_{BES+Chlo}$ on days 57 and 85 and a pulse of chloroform on day 73 of operation.

Routine analysis

Chemical oxygen demand (COD) and volatile suspended solids (VSS) were determined according to Standard Methods (APHA, 1989). Biogas flow rate was measured by a *Ritter Milligascounter* (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany). Hydrogen and methane content of gas was determined by gas chromatography. Hydrogen was measured using a Hayesep Q column

(80/100 mesh) and a thermal conductivity detector (Varian 3300 Gas Chromoatograph), with nitrogen (30 mL min^{-1}) as the carrier gas; injector, detector, and column temperatures were 120, 170, and 35ºC, respectively. Methane was measured using a *Porapack Q* column (100 - 180 mesh) and a thermal conductivity detector, with helium (30 mL min^{-1}) as the carrier gas; temperatures of the detector, injector and oven were 110, 110 and 35ºC, respectively. Volatile fatty acids (VFA), lactic acid, ethanol, L-arabinose, and glucose were determined by high performance liquid chromatography (Jasco, Japan) using a *Chrompack* column (6.5 x 30 mm^2). Sulphuric acid (0.01 N) at a flow rate of 0.7 mL min^{-1} was used as mobile phase; column temperature was set at 60 ºC. After eluting compounds were sequentially detected using two detectors: a UV detector at 210nm for VFA and lactic acid, and a RI detector for measuring ethanol, L-arabinose and glucose.

Biomass characterization

Granules after heat treatment (S_{HEAT}), granules after the contact with BES (S_{BES}), granules after the contact with BES+Chloroform ($S_{BES+Chlo}$), as well as, samples withdrawn from the reactors according to table 5.2 were stored at -18ºC until use for molecular analysis and at 4ºC for image analysis. An additional sample collected at day 11 from R_{BES} and $R_{BES+Chlo}$ was included for image analysis.

Morphological properties

Sludge sampling and processing for image analysis

Sludge sampling and processing were performed according to Araya-Kroff et al., (2004). Biomass was collected from the reactors using a wide bore tube connected to a 100 mL syringe avoiding mechanical stress. VSS were determined for all the collected samples.

Image analysis of granular sludge was done using diluted biomass samples. Optimal dilution was selected as the lowest dilution that enabled the maximum

percentage of objects that were recognized by the software. The percentage of recognition is the ratio between the area of objects that are completely inside the image and the total area of objects in the image including those that are at the boundaries and cannot be completely recognized. Depending on sludge concentration and size of aggregates, the optimal dilution determined for the samples was 1/5 and 1/10.

Image acquisition and analysis

Three sets of about 100 images were acquired for each sample in order to optimise image quality for detection and quantification of free and protruding filaments, micro-aggregates (equivalent diameter (Deq) < 0.2 mm) and macro-aggregates (Deq ≥ 0.2 mm). Images used to quantify macro-aggregates were acquired through visualisation on a SZ 40 stereo microscope (Olympus, Tokyo) with 15 x magnification. Images used to quantify filaments and micro-aggregates were acquired through phase contrast and bright field, respectively, on a Diaphot 300 microscope (Nikon Corporation, Tokyo) with 100 x magnification. All the images were digitized and saved with a CCD AVC D5CE grey scale video camera (Sony, Tokyo) and a DT 3155 frame grabber (Data Translation, Marlboro) with 768 x 576 pixel size with 8 bits (256 grey levels) by Image Pro Plus (Media Cybernetics, Silver Spring). For the filaments and micro-aggregates, a volume of 35 µL from the diluted sample was dispensed on a slide and covered with a 24x24 mm cover slip for visualization and image acquisition. This volume was exactly enclosed by the cover slip. Each image corresponded to a volume of 0.0499 µL. For the macro-aggregates an arbitrary volume was transferred to a Petri dish for visualization and image acquisition. Around 100 images per sample were acquired. Image acquisition of the micro-aggregates and filaments was obtained by three parallel horizontal passages along the slide at ¼, ½ and ¾ of its height for a total of 24 images per slide. With respect to the macro-aggregates, the Petri dish was thoroughly screened, from left to right and from top to bottom, and the VSS present in the Petri dish were measured after visualization and image acquisition. For each magnification, the pixels were converted to

metric dimensions using a micrometer. Image processing and analysis was done by means of three programmes developed in Matlab (The Mathworks, Inc., Natick), for filaments, micro, and macro-aggregates already reported (Abreu et al., 2007; Amaral et al., 2004; Costa et al., 2007; Costa et al., 2009). Different morphological parameters were calculated.

The filaments program was used to determine:

The specific total filaments length according to the follow equation:

$$L_{spec} = \frac{L}{V_{field}} \quad (1)$$

where V_{field} is the volume (μm^3) corresponding to the field of view (i.e., the image), L is the filaments length calculated according to the follow equation:

$$L = N * 1.222 * F_{cal} \quad (2)$$

where N is the number of pixels of the skeletonised filament and F_{cal} is the calibration factor ($\mu m\ pixel^{-1}$). The calibration factor 1.222 is used in order to homogenize the different angles of filaments (Walsby and Avery, 1996). Filaments length corresponded to filaments disperse on the bulk and attached to the aggregate with one free extremity (protruding filaments).

The micro and macro aggregates program was used to determine:

The total area (A) occupied by the aggregates in each image from which the specific area occupied by aggregates is calculated:

$$A_{spec} = \frac{A}{V_{field}} \quad (3)$$

Morphological parameters representing the dynamic evolution of filaments and aggregates inside the reactors as total filaments length per VSS (TL/VSS) and VSS per total projected area of aggregates (VSS/TA) were calculated as:

$$\frac{TL}{VSS} = \frac{L_{spec}}{VSS} \quad (4)$$

$$\frac{VSS}{TA} = \frac{VSS}{A_{spec}(<0.2mm) + A_{spec}(\geq 0.2mm)} \quad (5)$$

where VSS are the volatile suspended solids present in each sample and A_{spec} < 0.2mm and A_{spec} ≥ 0.2mm are the specific aggregates area for aggregates with Deq < and ≥ 0.2mm, respectively.

Microbial community

DNA Extraction, PCR and DGGE

Total genomic DNA was extracted from approximately 500µL of sample by using a FastDNA SPIN kit for soil (Qbiogene, Carlsbad, CA, USA) and maintained at 20ºC. Bacterial 16S rRNA genes were selectively amplified for cloning using primers Bact27-f (5'-GTT TGA TCC TGG CTC AG-3') and Uni1492-r (5'-CGG CTA CCT TGT TAC GAC-3') (Yu and Morrison, 2004). For DGGE analysis, DNA fragments of 456 base pairs were amplified by PCR using the primer set of 954GC-f (5'-GCA CAA GCG GTG GAG CAT GTG G-3') plus GC-Clamp (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3') and 1369-r (5'- GCC CGG GAA CGT ATT CAC CG-3'), targeting the V6 to V8 regions of bacterial 16S rRNA (Sanguinetti et al., 1994). The size of the obtained PCR products was checked by comparison with appropriate size and mass standard (MBI Fermentas, Vilnius, Lithuania), by electrophoresis on an 1% (w/v) agarose gel and ethidium bromide staining. Gels ran at a constant voltage of 100 V in an agarose gel electrophoresis system (Mupid-EX, Belgium). Nucleic acids were detected using an UV transilluminator (BioRad).

DGGE analysis of the amplicons was done by using the DCode system (Bio-Rad, Hercules, CA, USA). PCR products were electrophoresed in a 0.5x Trisacetate-EDTA buffer for 16h at 85V and 60ºC on polyacrylamide gel (8%) containing a linear gradient ranging from 30% to 60% denaturant. The silver staining of DGGE

gels was performed as previously described by (Hane et al., 1993). DGGE gels were scanned at 400 dpi and the DGGE profiles compared using the Bionumerics 5.0 software package (Applied Maths, Belgium). Similarity indices (Si) of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product-moment correlation (Cole et al., 2003). Community shifts were described as changes in the DGGE profiles of the partial 16S rDNA amplicons.

Shannon Wiener diversity indices (H) were calculated on the basis of the intensities of the bands on the gel tracks, as judged by peak height in the densitometric curves, according to the equation: $H = - \Sigma (Pi \ln(Pi))$ where, H is the diversity index and Pi is the importance probability of the bands in a lane ($Pi=ni/N$, where ni is the height of an individual peak and N is the sum of all peak heights in the densitometric curves).

Cloning and Sequencing

PCR products obtained with the primers set Bact27f and 1492r were purified using the Nucleo Spin Extract II kit (Clontech Laboratories), ligated into pGEM-T vector using the pGEM Easy Vector Systems kit (Promega),and introduced into competent E. coli ®10G & 10GF´ (Lucigen® Corporation) according to the manufacturer's instructions. Positive transformants were selected (blue-white screening) and grown in appropriate media supplemented with ampicillin. After cell lysis, plasmids were amplified using the primer set PG1f (5'-TGG CGG CCG CGG GAA TTC-3') and PG2r (5'-GGC CGC GAA TTC ACT AGT G-3') and the obtained PCR products analyzed in agarose gel (1%) in order to select clones with insert fragments of the correct size. Amplicons of the correct size were screened by amplified ribosomal DNA restriction analysis (ARDRA), using the restriction enzymes MspI, CfoI, and AluI (Promega). The restriction fragments were analyzed by electrophoresis in a 2% (w/v) agarose gel and visualized with ethidium bromide. Plasmids of selected transformants, with different ARDRA patterns and corresponding to predominant bands in the DGGE community fingerprint, were purified (Nucleo Spin Extract II kit) and subjected to DNA sequence analysis.

Sequencing reactions were performed at BIOPREMIER (Lisbon, Portugal) using pGEM-T vector-targeted sequencing primers SP6 (5'-GAT TTA GGT GAC ACT ATA G-3'), T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and internal specifically-tailored primers, when needed.

Phylogenetic analysis

16S rRNA gene partial sequences were assembled using the CAP application included in the BioEdit v7.0.9 software package. Consensus sequences were checked for potential chimera artifacts by the CHECK_CHIMERA program of the Ribosomal Database Project II (Cole et al., 2003) and sequences determined to be chimeras were removed from further analysis. Similarity searches for the assembled 16S rRNA gene sequences were performed using the NCBI BLAST search program within the GenBank database (Thompson et al., 1994). Phylogenic assignment of the sequences to higher order taxa was performed using the RDP Naive Bayesian Classifier.

Alignment of the 16S rRNA sequences was performed by using the FastAligner v1.03 tool of the ARB program package (Ludwig et al., 2004). The resulting alignments were manually checked and corrected when necessary, and unambiguously aligned nucleotide positions were used for the construction of a 16S rRNA gene-based phylogenetic tree by using the FastDNAMI method (Olsen et al., 1994). Phylogenetic placement was performed in comparison with reference sequences, with Felsenstein correction and application of appropriated filters at the respective phylum level.

Nucleotide sequence accession numbers

Nearly complete 16S rRNA sequences of the 16S rRNA gene clones were deposited in the GenBank database under accession numbers GU907811 to GU907825.

5.3 Results

Hydrogen production in continuous EGSB reactors

Hydrogen recovery **in R_{Heat},** inoculated with heat-treated granular sludge, was minor during the first operation period (HRT 20h) (Figure 5.1a). Acetate and butyrate were the main VFA measured in the bulk and methane was not detected, which indicates that produced hydrogen was most likely being channeled to homoacetogenesis. Homoacetogenic activity on H_2/CO_2 was further confirmed in batch experiments (data not shown). A peak of lactic acid (2047 mg L^{-1}) was observed at the end of the first operation period, coincident with a severe decrease in n-butyrate and acetate concentration. After setting the HRT at 12 h, lactic acid concentration decreased and, simultaneously, n-butyrate concentration and hydrogen production rate increased to values of up to 1000 mg L^{-1} and 1100 mLH_2 L^{-1} d^{-1}, respectively. This high hydrogen production rate was transient and, after 10 days, decreased to negligible values. Coincidently, acetate concentration rose up to a maximum of 2000 mg L^{-1}. Propionate concentrations during the second operation period of R_{Heat} increased to up to 800 mg L^{-1}. Higher propionate formation, coupled with homoacetogenesis, might justify the absence of hydrogen production after day 50 of operation.

R_{BES} and $R_{BES+Chlo}$ showed unstable hydrogen production and apparent homoacetogenesis during the first period of operation (HRT 20h) (Figure 5.1 b, c). During this first period, ethanol concentration increased up to 400 mg L^{-1}, in both reactors (data not shown), and butyrate concentration decreased from approximately 1100 mg L^{-1} to nearly 500 mg L^{-1} on average. After decreasing HRT to 12h, an increase on propionate concentration was observed in both reactors, up to values of 300 mgL^{-1} and 650 mgL^{-1} in R_{BES} and $R_{BES+Chlo}$, respectively. Homoacetogenesis was apparently taking place in the two reactors, although more strongly in R_{BES} where acetate peaked at a value as high as 2044 mg L^{-1} (Figure 5.1 b). Addition of a BES pulse to R_{BES} caused a decrease on acetate concentration to about 600 mg L^{-1}, but lactic acid concentration increased to 600 mg L^{-1}. Hydrogen production in R_{BES} restarted at day 72, when lactic acid and acetate concentrations were nearly at the same concentration (i.e. 600 mg L^{-1}).

From day 72 on, stable hydrogen production was observed in R_{BES} with an average hydrogen production rate of 700 ± 200 $mLH_2L^{-1}d^{-1}$. Hydrogen production in $R_{BES+Chlo}$ was very low throughout all the operation time (Figure 5.1 c) Additional pulses of BES and chloroform did not prompt hydrogen production.

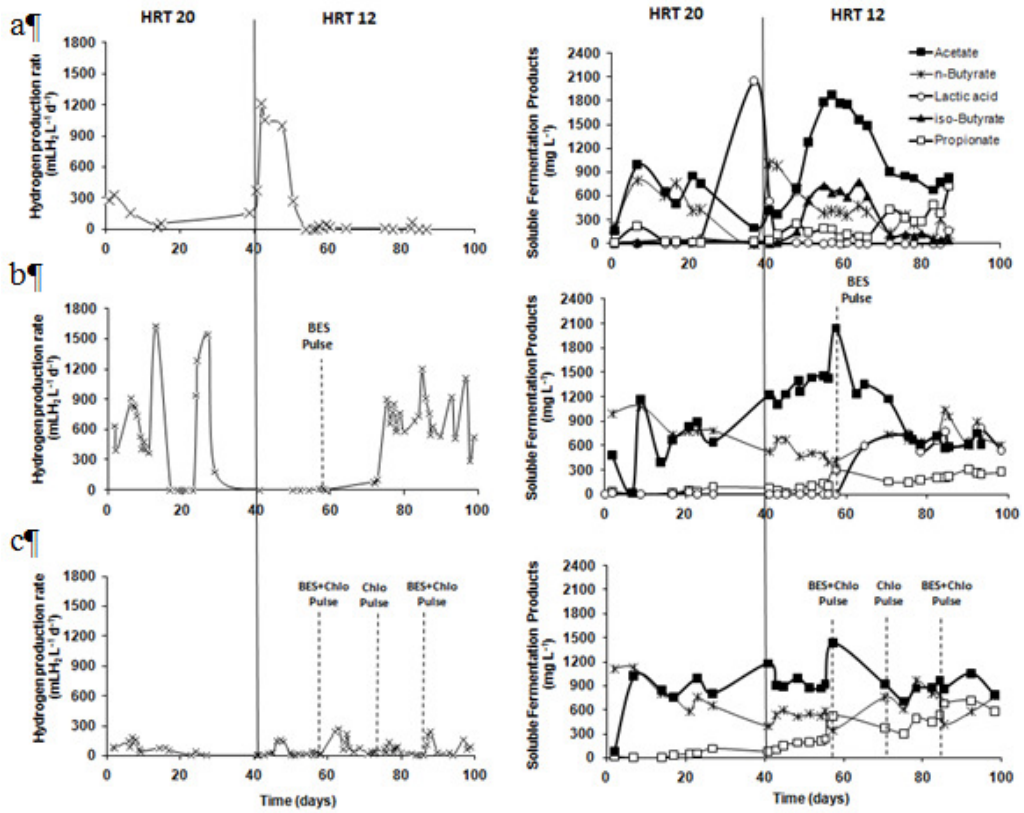


Figure 5.1. - Time course of hydrogen production rate and soluble fermentation products profile in R_{Heat} (a), R_{BES} (b) and $R_{BES+Chlo}$.(c). Pulses of BES and chloroform applied, as well as, the hydraulic retention time (HRT) set at each operation period are signalled in the figure.

Morphological properties of granular sludge

Image analysis was used to quantify changes in morphological properties of the granular sludge subjected to the different pretreatments. Total free filaments length per VSS (TL/VSS) in the three reactors increased during the first operational period (TRH 20 h) (Figure 5.2 a). This structural change was accompanied by a decrease in the amount of VSS per total projected area

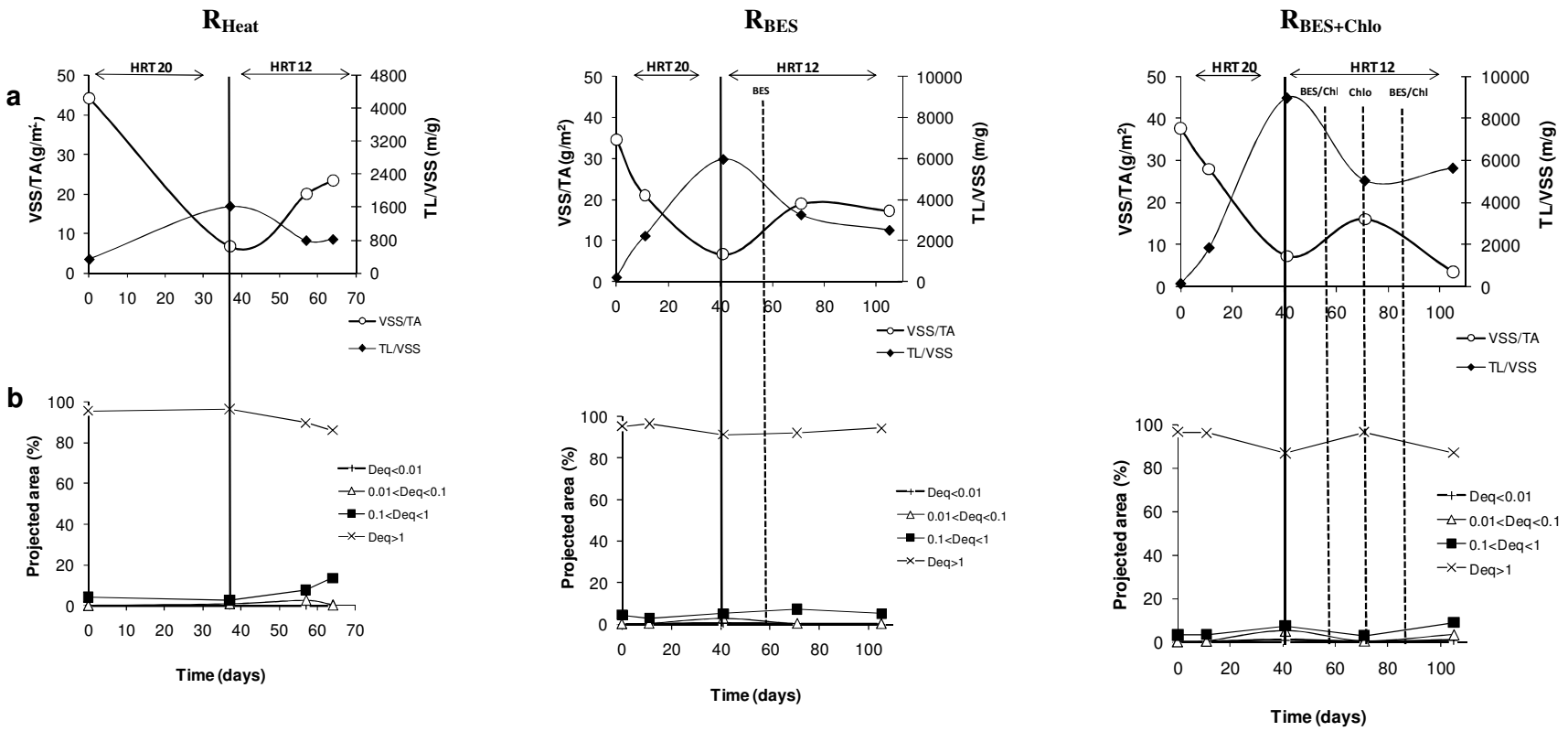


Figure 5.2. - Time course of R_{Heat}, R_{BES} and $R_{BES+Chlo}$ morphological parameters; (a) apparent density measured by mg VSS per total area of aggregates (VSS/TA) and total filaments length per VSS (TL/VSS); (b) percentage of total projected area of aggregates within different equivalent diameters (Deq) ranges.

(VSS/TA), indicating the release of filaments from the granules. Granules did not fragment in R_{Heat} during the first period of operation, as shown by the maintenance of the % of projected area of aggregates with D_{eq} > 1mm (Figure 5.2 b). This was not the case for sludges in R_{BES} and $R_{BES+Chlo}$, where a decrease in the % of projected area of aggregates with D_{eq} > 1mm was observed.

After changing the HRT to 12 h, TL/VSS decreased and VSS/TA of aggregates increased, in all the reactors (Figure 5.2 a). R_{Heat} showed the highest increase in VSS/TA, achieving 24 g m^{-2} at the end of operation. In this reactor, % of projected area of aggregates with D_{eq} > 1mm decreased from 96 to 88 %, with consequent increase of the fraction of aggregates with D_{eq} between 0.1-1 mm. In R_{BES}, TL/VSS decreased (to approximately 2700 m g^{-1}) and VSS/TA increased (up to 18 g m^{-2}). Addition of a BES pulse did not affect the integrity of granules and the % of projected area of aggregates with D_{eq} > 1mm kept a slightly increasing trend (Figure 5.2 b). In $R_{BES+Chlo}$ TL/VSS increased (to approximately 5900 m g-1) and a highly decrease in VSS/TA (to approximately 2 g m^{-2}) was observed after the second pulse of BES+Chloroform. These changes were concurrent with granules fragmentation. Original images of filaments and macro aggregates, and correspondent binary images, obtained at the end of the operation of each reactor are shown in Figure 5.3 as an example.

Microbial diversity

Pretreated sludges ($S_{HEAT,}$ S_{BES} and $S_{BES+Chlo}$), and samples withdrawn from the reactors along time, were analyzed regarding their bacterial diversity and composition Table 5.1.

DGGE profiles generated for each sludge sample were compared in terms of diversity index (H) and similarity index (SI). S_{HEAT} presented a lower diversity (H = 0.31) than the chemically treated granules (H = 1.43 and H = 1.20 for S_{BES} and $S_{BES+Chlo}$, respectively) (Figure 5.4).

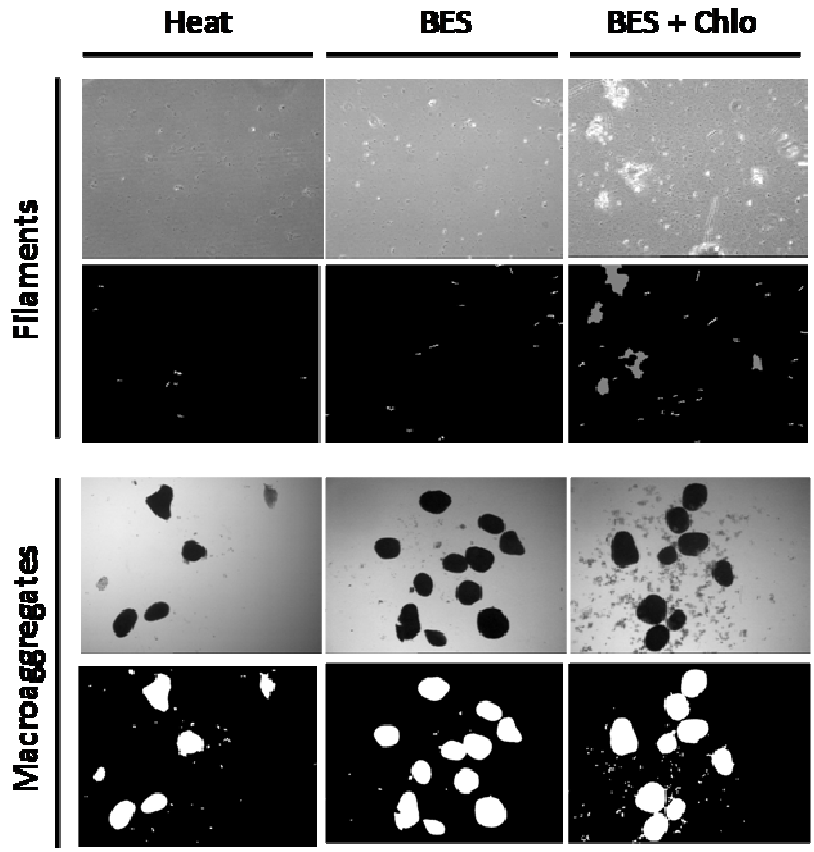


Figure 5.3. - Example of original images of filaments with a magnification of 100x and macroaggregates with magnification of 15x and the respective final binary images.

Shifts in the bacterial composition of reactor sludge samples during the first operation period were pronounced, with similarity indices between the inoculum sludges and samples collected on day 40 (end of the first operational period, samples $R_{Heat}1$, $R_{BES}1$ and $R_{B+C}1$) lower than 1%.

Bacterial diversity in S_{Heat} was very low (H=0.31), but increased with time; at end of operation, the diversity index in R_{Heat} was 5-fold higher than initially (Figure 5.4). Bacterial diversity in $R_{BES+Chlo}$ was not significantly affected (H=1.26 and H=1.28 for $R_{B+C}1$ and $R_{B+C}2$, respectively), until the addition of a second pulse of chloroform that caused a drop in the bacterial diversity with an H value of 0.72 for $R_{B+C}3$. The most stable bacterial diversity was observed in R_{BES}, with an average overall diversity index of 1.34 ± 0.10.

Further characterization of the bacterial community present in R_{BES} was preformed. The dominant bacterial ribotypes displayed in the DGGE profiles along the R_{BES} operation were found to be closely related to *Clostridium* species (*Clostridium sp* (99% identity), *Clostridium ljungdahlii* (99%), *Clostridium drakei* (94%), *Clostridium ragsdalei* (100%) and uncultured bacterium sequences belonging to *Clostridiaceae* and *Ruminococcaceae* families. 16S rDNA clone sequences were used to construct the phylogenetic tree as given in Figure 5.5.

Table 5.1. - Samples collected for DGGE analysis from $R_{Heat,}$ R_{BES} and $R_{BES+Chlo}$ and correspondent operational conditions at time of sampling. OLR corresponds to organic loading rate and HRT to hydraulic retention time

Treatments	Samples	Time (d)	HRT (h)	OLR (kgCOD m^{-3} d^{-1})
Heat	$R_{heat}1$	40	20	6.40
	$R_{heat}2$	57	12	10.66
	$R_{heat}3$	64	12	10.66
BES	$R_{BES}1$	40	20	6.40
	$R_{BES}2$	73	12	10.66
	$R_{BES}3$	97	12	10.66
BES+Chloro	$R_{B+C}1$	40	20	6.40
	$R_{B+C}2$	73	12	10.66
	$R_{B+C}3$	98	12	10.66

5.4 Discussion

In the last years, different methods have been studied for the effective development of anaerobic granular sludge for hydrogen production. All these methods have been studied in terms of hydrogen production and yields (Hu and Chen, 2007). In the present study, we evaluated the effect of heat treatment and two chemical treatments (contact with BES and with BES+Chloroform) on the

macro and micro-scale morphology and microbial community structure in the operation of high-rate anaerobic reactors.

Heat treatment was inefficient for inhibiting propionate formers and homoacetogenic microorganisms, as previously reported by other authors (Oh et al., 2003). Heat caused immediate and strong decrease in bacterial diversity of the granular sludge (Figure 5.4), likely due to the elimination of H_2-consumers but also due to suppression of other bacteria. In fact, heat acts as a non-selective pressure enriching microbial sludges on spore-forming bacteria and not on specific physiological groups. Methanogens are normally eradicated, but homoacetogens can resist because some can form spores (Tanner et al., 1993).

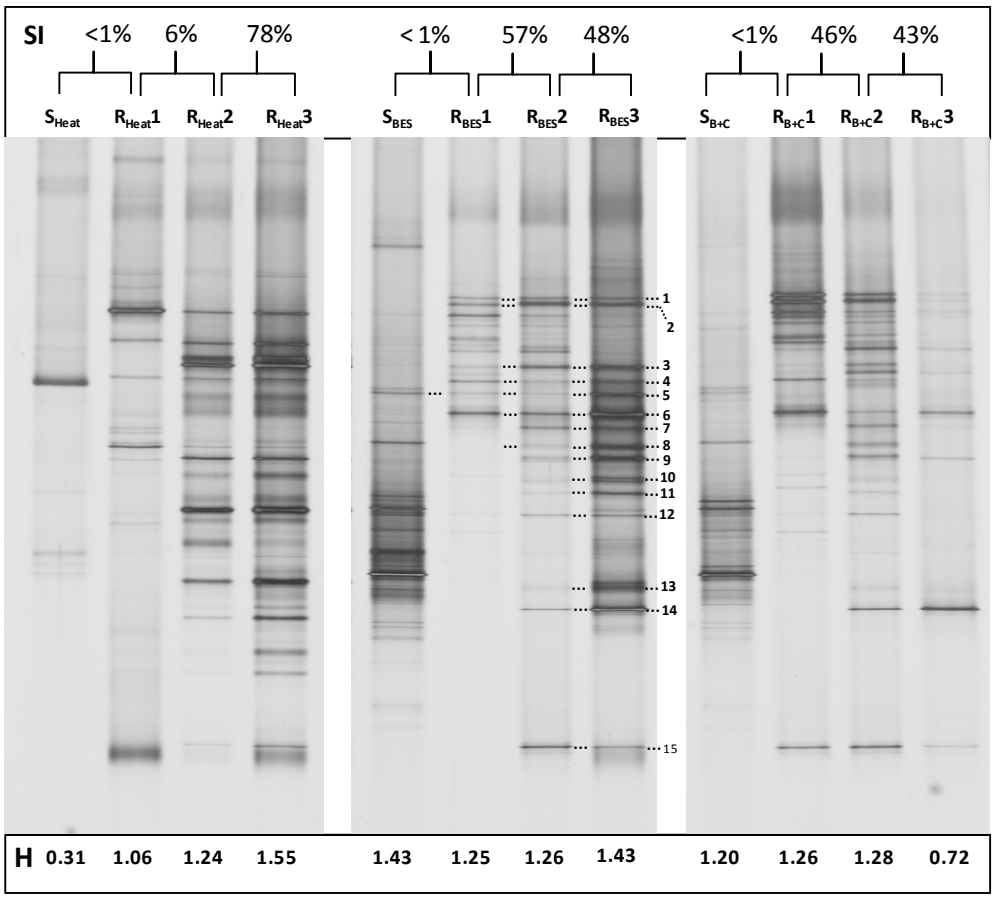


Figure 5.4. - DGGE profiles of granules after heat treatment (S_{HEAT}); granules after the contact with BES (S_{BES}); granules after the contact with BES+Chloroform ($S_{BES+Chlo}$) and samples withdrawn from R_{Heat}, R_{BES} and $R_{BES+Chlo}$ along the operation according to Table 5.1. Similarity (SI) and Diversity (H) indexes.

Chloroform is thought to be an inhibitor for homoacetogens, and it could actually inhibit some homoacetogenic activity; acetate concentrations in $R_{BES+Chlo}$ were, in general, lower than in R_{Heat} and R_{BES}. Nevertheless, an increase on propionate formation in the presence of chloroform impaired a good overall hydrogen yield. After day 72 the most stable hydrogen production was observed in R_{BES} (average hydrogen production rate of 700±200 $mLH_2L^{-1}d^{-1}$). Hydrogen production in this reactor was linked to the consumption of acetate and lactate. Production of hydrogen, carbon dioxide and butyrate from acetate and lactic acid has been reported for *Clostridium diollis* (Matsumoto and Nishimura, 2007). These results demonstrate that some routes for hydrogen production are not only dependent on the presence of hydrogen producers but also on the co-metabolism of the whole microbial community.

Besides the microbial and metabolic aspects, granular sludge should maintain the morphological properties and integrity essential for the operation of high-rate reactors. When granular sludge deteriorates, a release of filaments and/or fragments can occur, simultaneously with the decrease in density and/or size of the aggregates. Sludge washout is usually an immediate consequence, due to the low hydraulic retention times usually applied in high-rate reactors, leading ultimately to process failure.

For the three studied inocula pretreatments, an increase of filaments release and a decrease of granules density were verified during the first period of operation (TRH 20h), likely linked to the washout of microorganisms purged by the selective process. This is supported by the high VSS content detected in the effluent, as well as, the considerable shift on the microbial population observed in all reactors during this period of operation (SI<1%, Figure 5.4).

During the second period of operation, bacterial community in R_{Heat} showed lower fluctuations and seemed to stabilize. Simultaneously, considerable changes occurred in terms of morphological properties resulting in a significant increase on the apparent granules density (Figure 5.2 and Figure 5.4). Contrarily, considerable microbial population modifications were observed along R_{BES} operation even though granules apparent density has been maintained roughly

at 20 g m^{-2} and significant filaments release was not observed. This means that some organisms were being released from the aggregated structure of granular sludge and replaced by others, but this dynamic did not affect the granules morphology and density. Bacterial diversity in $R_{BES+Chlo}$ decreased notably after the addition of a second chloroform pulse (Figure 5.4). This fact was coincident with a considerable decrease of granules density (VSS/TA of 2 g m^{-2}) and increase of filaments release (TL/VSS of 5900 m g^{-1}) (Figure 5.2), concurrent with the fragmentation of granules. Granule fragmentation was also observed in R_{Heat} during the second period of operation. Opposite behavior was observed in $R_{BES,}$ this system maintained along the operation time the % of projected area of aggregates with an equivalent diameter larger than 1mm.

The chemical pretreatment strategy using BES+Chloroform lead to granules deterioration, but the application of BES only, showed higher hydrogen production potential and did not affect significantly the granules integrity. This strategy revealed higher potential for high-rate systems, since it was more capable of maintaining higher biomass concentration under high dilution rates without biomass washout.

Results obtained for the bacterial composition in R_{BES} are in agreement with several studies that have suggested that *Clostridium*-like bacteria are the dominant microorganisms present in H_2-producing systems (Lay et al., 1999).

Interestingly, 16S rDNA sequences phylogeneticaly positioned within the *Ruminococcaceae* family were also found in R_{BES}. Recently a novel bacterium from the *Ruminococcacea* family, *Hydrogenoanaerobacterium saccharovans*, was isolated from a H_2-producing UASB reactor (Song and Dong, 2009).

This non spore former organism can use different mono-, di- and oligosaccharides to produce acetate, ethanol, hydrogen and carbon dioxide. The fact that some hydrogen producers are non spore formers highlights the importance of using different methods, for directing granular sludge for optimal hydrogen production, as an alternative to the methods based on the stimulation of spore formation.

The presence of organisms closely related to *Clostridium ljungdahlii* and *Clostridium autoethanogenum* might possibly explain the high acetate concentrations and low hydrogen production observed in R_{BES} before day 72. These acetogenic organisms can use several substrates to produce acetate including hydrogen and carbon dioxide, decreasing the overall hydrogen production rate (Abrini et al., 1994; Tanner et al., 1993).

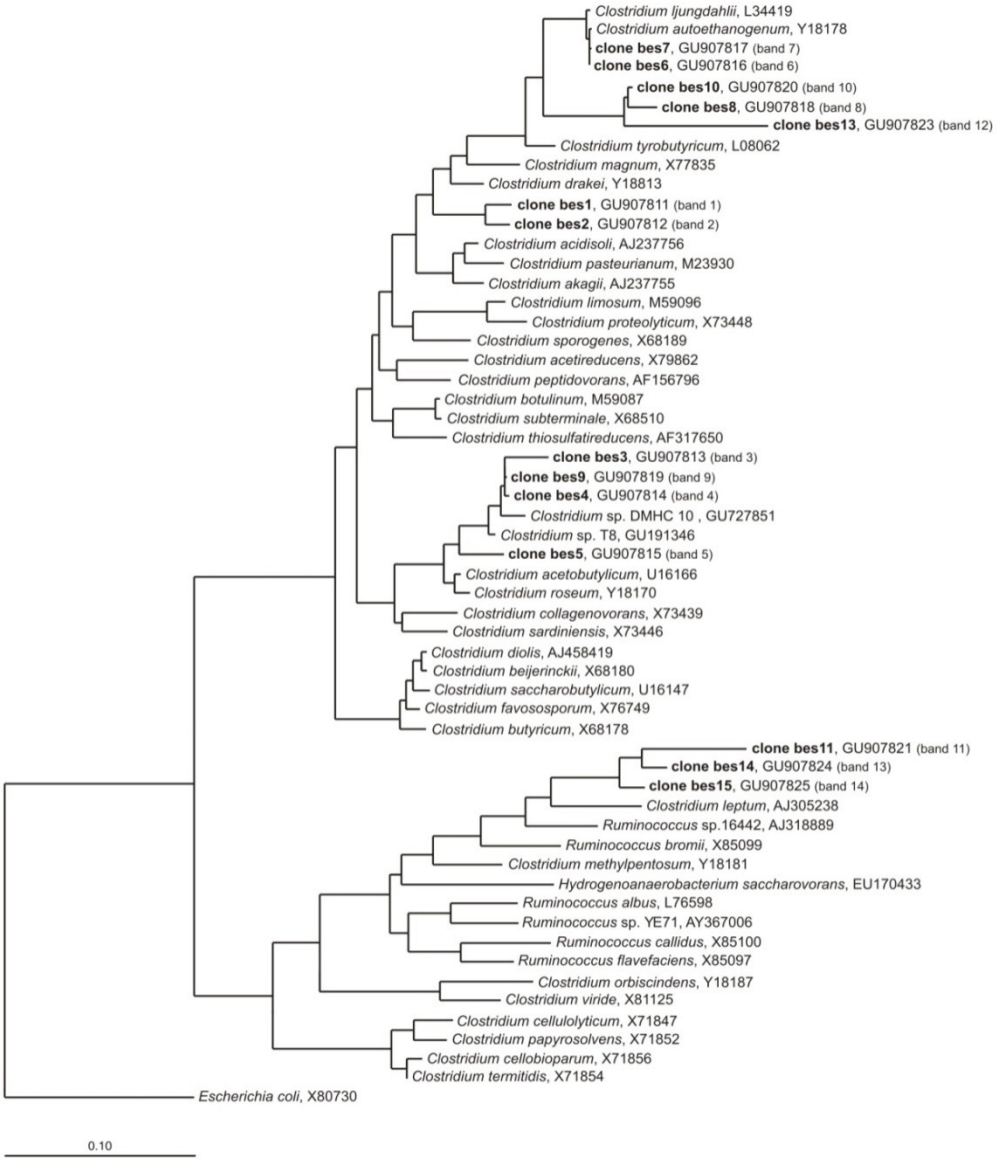


Figure 5.5. - FastDNAMI tree of partial 16S rRNA gene sequences from R_{BES} clones and closest relatives imported from NCBI database. GenBank accession numbers of 16S rRNA gene sequences used to construct the tree are shown; DGGE bands corresponding to each of the sequenced clones is given in parentheses. Bar corresponds to 10% sequence divergence.

5.5 Conclusions

In conclusion, this study showed that strategies used for suppress the activity of H_2-consuming microorganisms affect both micro- and macro scale structure and microbiology of granular sludge, which can be incompatible with the long term operation of high-rate reactors. Comparing with the other strategies studied, pretreatment and subsequent pulses with BES revealed to be the strategy with higher potential for high-rate reactors start-up and further stable continuous operation, at mesophilic conditions. This technique was effective in extinguishing the methanogenic hydrogenotrophic activity and did not extensively affect macro- and microstructure of the granules. Homoacetogenic activity, however, could not be completely inhibited indicating that further studies need to be performed in order to select an effective strategy for complete suppression of this group.

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Engineered heat treated methanogenic granules: a promising biotechnological approach for extreme thermophilic biohydrogen production

Chapter 6

Abstract

In the present study, two granular systems were compared in terms of hydrogen production rate, stability and bacterial diversity under extreme thermophilic conditions (70ºC). Two EGSB reactors were individually inoculated with heat treated methanogenic granules (HTG) and HTG amended with enrichment culture with high capacity of hydrogen production (engineered heat treated methanogenic granules - EHTG), respectively. The reactor inoculated with EHTG (R_{EHTG}) attained a maximum production rate of 2.7 L H_2 $L^{-1}d^{-1}$ in steady state. In comparison, the R_{HTG} containing the HTG granules was very unstable, with low hydrogen productions and only two peaks of hydrogen (0.8 and 1.5 L H_2 $L^{-1}d^{-1}$). The presence of active hydrogen producers in the R_{EHTG} system during the reactor start-up resulted in the development of an efficient H_2-producing bacterial community. The results showed that "engineered inocula" where known hydrogen producers are co-inoculated with HTG is an efficient way to start up biohydrogen producing reactors.

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6.1 Introduction

The search for new alternative fuels is a priority and hydrogen is a promising clean energy alternative to fossil fuels, since water is the only reaction product of oxidation. Chemoheterotrophic ("dark") fermentation process has received much attention recently due to the fact that hydrogen can be generated continuously at high-rate from renewable organic materials (Das and Veziroglu, 2001). Dark fermentation generally results in a higher hydrogen production rate and higher bacterial growth than photofermentation (Levin et al., 2004). The possibility to produce H_2 without the demand of light energy is another advantage of dark fermentation, resulting in lower capital costs for at least small-scale production facilities (100-1000 L H_2h^{-1}) (Das and Veziroglu, 2001; Hawkes et al., 2007). Almost all studies of hydrogen production with mixed cultures have been performed under mesophilic (26-40ºC) or thermophilic (45-60ºC) conditions. Recently, fermentation under extreme-thermophilic conditions (70ºC), started to attract attention due to: much better pathogen destruction for residues coming from anaerobic fermentation process; lower risk of contamination with methanogenic organisms (van Groenestijn et al., 2002), higher rate of hydrolysis (Lu et al., 2008) and higher hydrogen yield (Kadar et al., 2004). Continuous processes for fermentative H_2 production can be divided into two major categories; namely, suspended systems and immobilized systems (Oh et al., 2004). Suspended systems allow better mass transfer between microorganisms and substrates, but have difficulty in maintaining a sufficient amount of H_2-producing bacterial population inside the bioreactor under low hydraulic retention time (Chen et al., 2001). Immobilized based systems have been used for hydrogen production since they are more capable of maintaining higher biomass concentration under high dilution rates without biomass washout (Zhang et al., 2008c).

In general, immobilized-cell techniques include surface attachment (Oh et al., 2004), self-flocculation (Kim et al., 2005) and gel entrapment (Wu et al., 2006) approaches. These techniques have been applied to produce H_2 continuously in fixed-bed (or packed-bed) bioreactors (Lee et al., 2003), granular-sludge bed

bioreactors (e.g., CIGSB) (Lee et al., 2004), trickling biofilter reactors (TBR) (Oh et al., 2004), up-flow anaerobic sludge blanket bioreactor (UASB) (Thong et al., 2008a) and fluidized bed bioreactors (FBRs) (Lin and Cheng, 2006). In granular-sludge systems, cell immobilization is achieved, under certain conditions, by self-aggregation of anaerobic microorganisms into granules, in the absence of a support material. A major drawback in granule based processes is the long startup period, which generally requires several months for the formation of hydrogen-producing granules (Mu and Yu, 2006). Engineering microbial mixed communities present in already formed anaerobic granules towards improved hydrogen production, by means of environmental pressure, is thus an attractive alternative to circumvent the long startup periods required for the development of hydrogen-producing granules. The bacterial community structures of hydrogen-producing systems have been extensively characterized. Diverse mesophiles and thermophiles affiliated with the classes *Clostridia* and *Bacilli* in the phylum Firmicutes have been reported (Wu et al., 2006). However, there are few studies on community structure of hydrogen-producing anaerobic microflora at temperatures higher than 60ºC (Thong et al., 2008a; Yokoyama et al., 2007).

The aim of the present work was to compare two different startup strategies for extreme thermophilic expanded granular sludge blanket (EGSB) reactor systems. The first EGSB reactor was inoculated with heat treated methanogenic granules (HTG), and the second with engineered heat treated methanogenic granules (EHTG), obtained by contact of HTG with an enriched hydrogen producing culture. The two strategies were compared in terms of hydrogen production rate, process stability and bacterial community structure. Synthetic medium used in this study contained glucose and arabinose, components of various plant biopolymers (hemicellulose and pectin) and lignocellulosic hydrolysates with a significant potential for biohydrogen production.

6.2 Materials and Methods

Source of inocula

Inoculum from H_2 producing laboratory-scale continuously stirred tank reactor (CSTR) treating household solid wastes (HSW) at 70^{o}C (Liu et al., 2006) was used to obtain enriched culture with high hydrogen production potential by repeated batch cultivation. The CSTR was fed with HSW at a hydraulic retention time of 2 days and organic loading rate of 11 g volatile solids $L^{-1}d^{-1}$ at 70°C for approximately one year, and was originally inoculated with the effluent from a mesophilic (38^{o}C) full-scale biogas plant treating a mixture of wastewater sludge and municipal solid wastes (Grindsted, Denmark).

Methanogenic granules were obtained from an upflow anaerobic sludge blanket (UASB) reactor used for treating brewery wastewater (Lisbon, Portugal).

Medium preparation and repeated batch cultivations

Basal anaerobic (BA) medium was used for enrichment and routine cultivation in batch, as described previously (Angelidaki et al., 2004), except that no L-cysteine hydrochloride was added, and the concentration of $Na_2S.9H_2O$ was increased to 0.25 g L^{-1}. The medium was supplemented with sterile solutions of glucose, arabinose, yeast extract, and peptone to final concentrations of $2gL^{-1}$, $2gL^{-1}$, $2gL^{-1}$ and 1 gL^{-1}, respectively. 40 mL medium was then dispensed in 100 mL serum bottles. The medium was flushed with nitrogen for 15 min to obtain anaerobic conditions and the pH was adjusted to 7.0. After inoculation with 4 ml inoculum from late exponential growth phase (OD_{660}=0.5), the serum bottles were incubated in dark at 70^{o}C. When the culture reached late exponential growth phase, it was used to make new inoculations in fresh BA medium with composition described above. Six repeated batch cultivation of the enriched mixed culture were performed.

Set-up and operation of the H_2-producing reactors

Pretreatment of granules

The methanogenic granules were boiled at 100ºC for 15 min (subsequently named, HTG) as described by (Lay et al., 1999). The continuous experiments were carried out in EGSB reactors (Figure 6.1) made of plexiglass with a height of 1.95 m and internal diameter of 21 mm. Total liquid volume was 1.30 L inclusive reaction-zone volume of 0.7 L and the superficial velocity was set at 10.0 m h^{-1} by means of an internal recirculation. The reactors were operated with a stable temperature of 70 ± 1 ºC by means of an external jacket for water circulation.

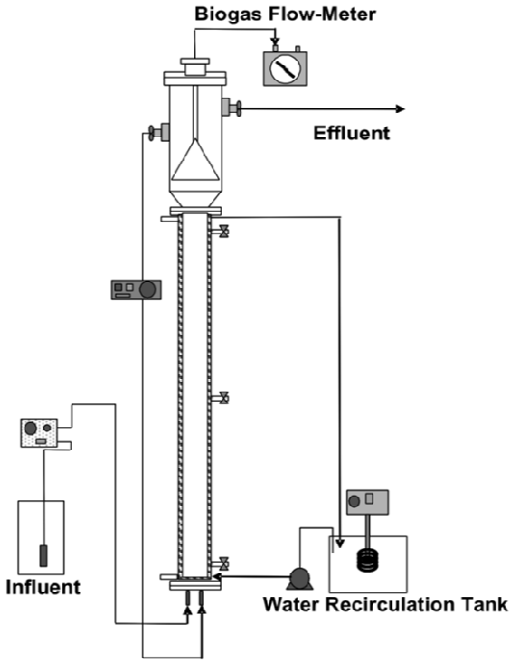

Figure 6.1. - EGSB reactor set-up.

One EGSB reactor, designated as R_{HTG}, was inoculated with 400 mL of HTG (47.6mgVSS g^{-1}). The second reactor, R_{EHTG}, was inoculated with 400 mL of HTG and 600 ml (OD_{660}=0.5) of the hydrogen producing enriched culture (obtained by repeated batch cultivation, as described above). After inoculation, R_{EHTG} was operated in a batch mode for 3 days only with recirculation in order to promote the contact between the culture and HTG surface. The recirculation flow rate was 20 mL min^{-1}. HTG amended with the enriched hydrogen culture were designated as EHTG (engineered heat treated methanogenic granules).

Thereafter, R_{HTG} and R_{EHTG} were operated continuously and fed with glucose and L-arabinose with ratio 1:1 (w/w) at final concentrations of 5 g COD L^{-1}. The feedstock was supplemented with macronutrients ($MgSO_4.7H_2O$: 30 g L^{-1}; KH_2PO_4: 28.3 g L^{-1}; NH_4Cl: 170 g L^{-1}). The macronutrients were added to the influent feed by addition of 0.6 mL g^{-1} COD fed. To give suitable alkalinity 2 g $NaHCO_3$ were added per liter of feed. The feedstock was stored at 4 °C, to minimize acidification.

During the first 7 days of continuous mode, R_{EHTG} was operated with a hydraulic retention time (HRT) of 27h. R_{EHTG} was fed with 2 g COD L^{-1} for 4 days and afterwards the feed concentration was increased to 5 g COD L^{-1}. HRTs of 14h, 8h and 6h were applied in R_{EHTG} and these changes were performed when steady state in hydrogen production rate (more than 3HRT with hydrogen production rate variation less than 5%) was achieved.

Analytical methods

The COD was determined according to Standard Methods (APHA, 1989). Biogas flow rate was measured by a *Ritter Milligascounter* (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany). Hydrogen content of the gas phase was determined by gas chromatography (GC) using a Hayesep Q column (80/100 mesh) and thermal conductivity detector (Varian 3300 Gas Chromatograph) with nitrogen (30mL min^{-1}) as the carrier gas. The injector, detector, and column temperatures were 120, 170, and 35ºC respectively. Methane content was determined by gas chromatography using a *Porapack Q* (100 - 180 mesh) column, with Helium as the carrier gas at 30 mL min^{-1} and thermal conductivity detector. Temperatures of the detector, injector and oven were 110, 110 and 35ºC, respectively. Volatile fatty acids (VFA), ethanol, lactic acid, L-arabinose, and glucose were determined by high performance liquid chromatography using an HPLC (Jasco, Japan) with a *Chrompack column* (6.5 x 30 mm^2); sulphuric acid (0.01 N) at a flow rate of 0.7mL.min^{-1} was used as mobile phase. Column temperature was set at 60 ºC. Detection of VFA and ethanol, lactic acid, L-arabinose, glucose was made sequentially with an UV detector at 210 nm and a RI detector, respectively.

Scanning electron microscopy observations

The surface structure of HTG and EHTG was observed by means of scanning electron microscopy (SEM) (Quanta 200F FEI, Oxford Instruments, Oxford). Sample preparation and fixation was preformed according to (Tay et al., 2001). The dried samples were sputter coated with Au-Pd (Sputter coater 208HR, Cressington) and finally observed by SEM.

PCR -DGGE

Representative granular sludge samples (mixture of granules, and suspended growing cells), collected from the reactors were stored at -18^{o}C until use. Total genomic DNA was extracted from approximately 500uL of sample by using a FastDNA SPIN kit for soil (Qbiogene, Carlsbad, CA, USA). The extracted DNA was maintained at -20^{o}C. 10-fold serial dilutions from extracted DNA were made and tested in the PCR to find the best concentration of template DNA that gives a good specific product. The 16S rRNA genes were amplified by PCR using a Taq DNA polymerase kit (Life Technologies, Gaithersburg, MD, USA), with primers targeting conserved domains. Bacterial 16S rRNA genes were selectively amplified for cloning using primers Bact27-f (5'-GTT TGA TCC TGG CTC AG-3') and Uni1492-r (5'-CGG CTA CCT TGT TAC GAC-3'). The thermocycling program used for amplification was as follows: pre-denaturation at 95°C for 5 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 40 s, and elongation at 72°C for 90 s; and pos-elongation at 72°C for 5 min. The reactions were subsequently cooled to 4°C. For DGGE analysis, DNA fragments of 456 base pairs were amplified by PCR using the primer set of 954GC-f (5'-GCA CAA GCG GTG GAG CAT GTG G-3') plus GC-Clamp (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3') and 1369-r (5'- GCC CGG GAA CGT ATT CAC CG-3'), targeting the V6 to V8 regions of bacterial 16S rRNA (Yu, et al. 2004). The programme for amplification was as described above but with 35 cycles and an annealing temperature of 56°C. The size of the obtained PCR products was checked by comparison with appropriate size and mass standard (MBI Fermentas, Vilnius,

Lithuania), by electrophoresis on an 1% (w/v) agarose gel and ethidium bromide staining. Gels ran at a constant voltage of 100 V in an agarose gel electrophoresis system (Mupid-EX, Belgium). Nucleic acids were detected using an UV transilluminator (BioRad).

DGGE analysis of the amplicons was done by using the DCode system (Bio-Rad, Hercules, CA, USA). PCR products were electrophoresed in a 0.5x Trisacetate-EDTA buffer for 16h at 85V and 60ºC on polyacrylamide gel (8%) containing a linear gradient ranging from 30% to 60% denaturant. Silver staining of DGGE gels was performed as previously described by (Sanguinetti et al., 1994). DGGE gels were scanned at 400 dpi and the DGGE profiles compared using the Bionumerics 5.0 software package (Applied Maths, Belgium). Similarity indices (Si) of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product-moment correlation (Hane et al., 1993). Community shifts were described as changes in the DGGE profiles of the partial 16S rDNA amplicons.

Shannon-Wiener diversity indices (H) were calculated on the basis of the intensities of the bands on the gel tracks, as judged by peak height in the densitometric curves, according to the equation: $H = - \Sigma (Pi \ln(Pi))$ where, H is the diversity index and Pi is the importance probability of the bands in a lane ($Pi=ni/N$, where ni is the height of an individual peak and N is the sum of all peak heights in the densitometric curves).

Cloning and Sequencing

PCR products obtained with the primers pair Bact27f and 1492r, described above, were further purified with the Nucleo Spin Extract II kit (Clontech Laboratories), ligated into the pGEM-T vector using the pGEM Easy Vector Systems kit (Promega),and introduced into competent *E. coli* ®10G & 10GF´ (Lucigen® Corporation), according to the manufacturer's instructions. Positive transformants were selected (by blue/white screening) and grown in appropriate media supplemented with ampicillin. After cell lysis, plasmids were amplified

using the primer set PG1f (5'-TGG CGG CCG CGG GAA TTC-3') and PG2r (5'-GGC CGC GAA TTC ACT AGT G-3') and the obtained PCR products were analyzed in agarose gel (1%) in order to select clones with right insert fragments. Amplicons of the correct size were screened by amplified ribosomal DNA restriction analysis (ARDRA), using the restriction enzymes MspI, CfoI, and AluI (Promega). The restriction fragments were analyzed by electrophoresis in a 2% (w/v) agarose gel and visualized with ethidium bromide. Plasmids of selected transformants, with different ARDRA patterns and corresponding to predominant bands in the DGGE community fingerprint, were purified (Nucleo Spin Extract II kit) and subjected to DNA sequence analysis. Sequencing reactions were performed at BIOPREMIER (Lisboa, Portugal) using pGEM-T vector-targeted sequencing primers SP6 (5'-GAT TTA GGT GAC ACT ATA G-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3'). Similarity searches for the 16S rRNA gene sequences with approximately 1400 base pair derived from the sludge clones were performed using the NCBI BLAST search program within the GenBank database.

Nucleotide sequence accession numbers

Sequences of the 16S rRNA gene clones were deposited in the GenBank database under accession numbers GU296463 to GU296478.

6.3 Results and Discussion

Reactors performance – EHTG *versus* HTG

Hydrogen production

R_{EHTG} showed a stable hydrogen production rate during the entire operational period and in each HRT, steady state was achieved (Figure 6.2 a). The maximum hydrogen production rate under steady state (2700 mL H_2 L^{-1} d^{-1}) was attained with HRT of 6h, although no significant difference was observed between HRT 6h and 8h. Hydrogen content in the produced biogas was stable during the entire operation and varied between 30 and 40% (Fig. 2b). R_{EHTG} achieved the highest

hydrogen yield (175 mL H_2 g^{-1} substrate) with HRT of 27h and feed concentration of 5g L^{-1} (Table 6.1). No methane was detected in the gas phase. In contrast, in R_{HTG}, almost no hydrogen production was observed during the entire operational period (Figure 6.3 a). Transient hydrogen production peaks were observed between days 13 and 18 (maximum rate of 800 mL H_2 L^{-1} d^{-1} under HRT of 13 h) and between days 30 and 36 (maximum rate of 1500 mL H_2 L^{-1} d^{-1} under HRT of 8h). During the rest of the operational period, hydrogen production rate was less than 100 mL H_2 L^{-1} d^{-1}. In addition, hydrogen content in R_{HTG} was less than 18% during the entire operational period, except in the periods that corresponded to peaks of hydrogen production (44 and 52%) (Figure 6.3 b). Significant hydrogen production and process stability was obtained in engineered heat treated granules system. The performance of R_{EHTG} was comparable to other studies with extreme thermophilic hydrogen producers. Kotsopoulos and co-authors reported hydrogen production rates ranging from 636 to 1100 mL H_2 L^{-1} d^{-1}, depending on start-up strategy, in a UASB reactor using 5g COD L^{-1} of glucose as carbon source, and HRT ranging from 26 to 24h. The inoculum was compost by autoclaved granules that functioned as carriers for immobilization of a mixed culture (Kotsopoulos et al., 2006). In the present study 1000 mL H_2 $L^{-1}d^{-1}$ was achieved with a HRT of 27h using the same feed concentration as these authors (Kotsopoulos et al., 2006) but more complex feed composition (mixture of glucose and arabinose (1/1)).

van Groenestijn and co-authors (van Groenestijn et al., 2009) fed a sucrose solution to a continuous reactor at a load 3.75 times higher than that used in our study. The 4.8 times higher hydrogen production rate obtained by these authors, was likely due to the simpler feed composition used (sucrose versus mixture of arabinose and glucose) and the inoculation with a pure culture of *Caldicellulosiruptor sacharolyticus*.

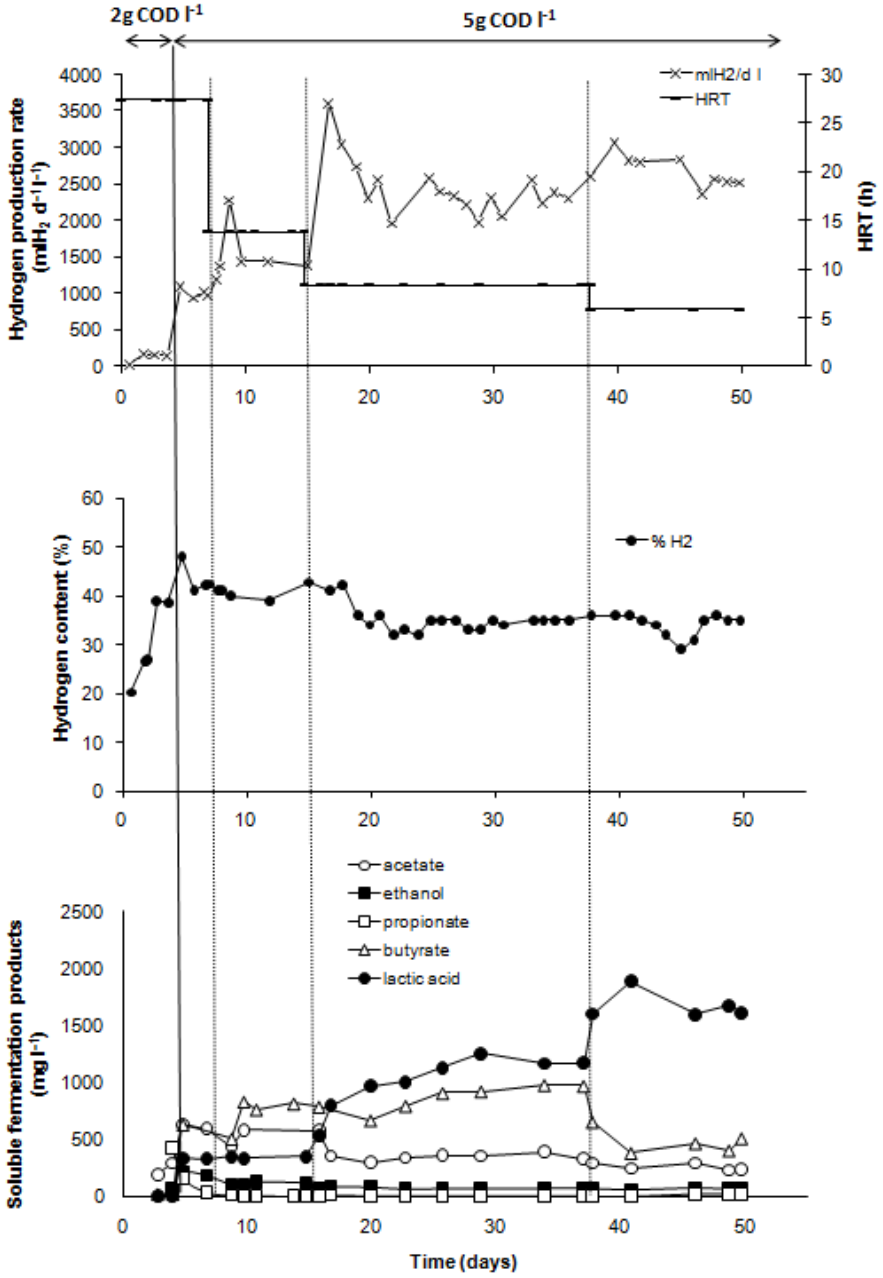

Figure 6.2. - Effect of HRT on the performance of R_{EHTG} (engineered heat treated granules system) (a) hydrogen production rate and HRT, (b) hydrogen content and (c) soluble fermentation products. Data represent average value from triplicate experiment, standard deviations were always within 5-10%.

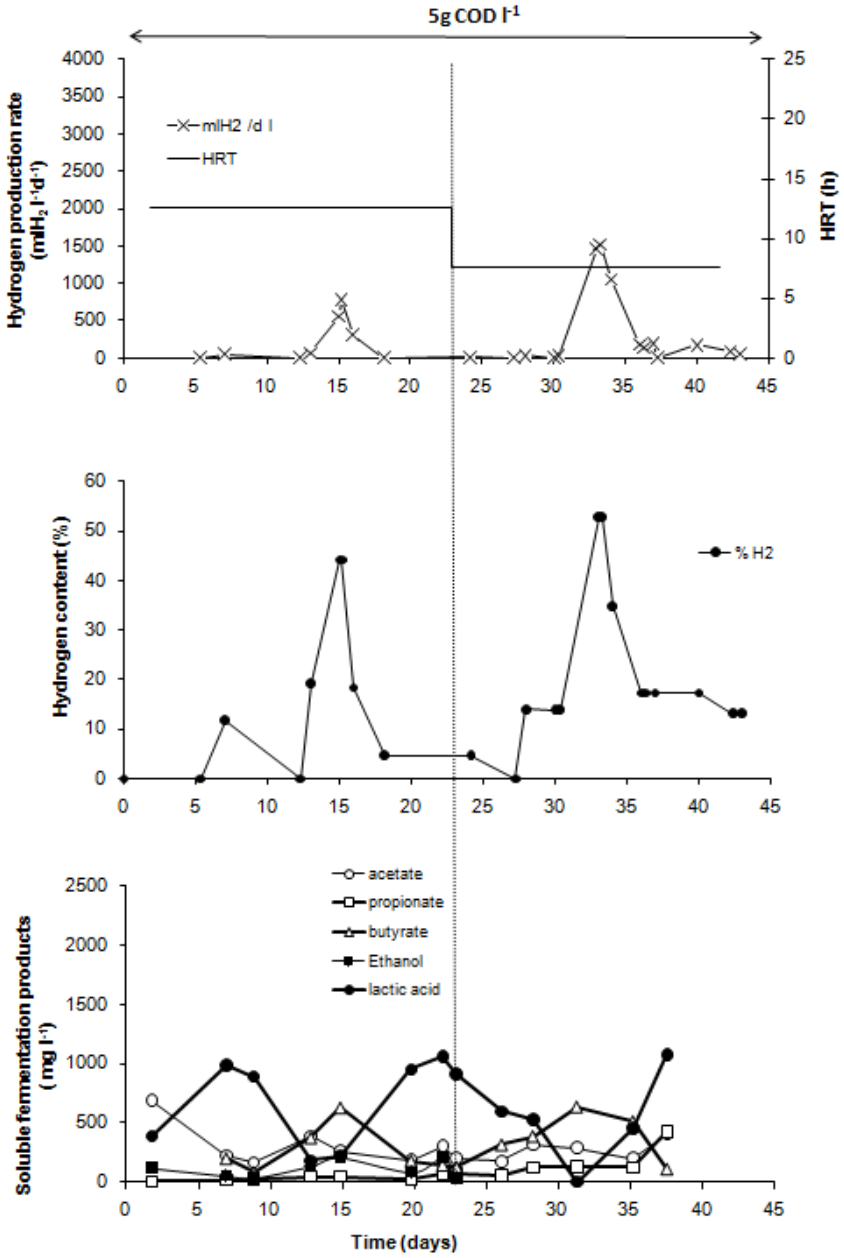

Figure 6.3. - Effect of HRT on the performance of R_{HTG} (self-formatted heat treated granules system) (a) hydrogen production rate and HRT, (b) hydrogen content and (c) soluble fermentation products. Data represent average value from triplicate experiment, standard deviations were always within 5-10%.

Sugars consumption

Glucose was totally consumed in both reactors, except in R_{EHTG} with application of HRT of 6h (Figure 6.4 and Table 6.1). Uptake of arabinose in R_{HTG} was less than in R_{EHTG} independently of the HRT applied (Table 6.1). In R_{EHTG}, the arabinose utilization was 2.5 times higher for the HRT of 14h and 1.6 times higher for the HRT of 8h compared to R_{HTG}.

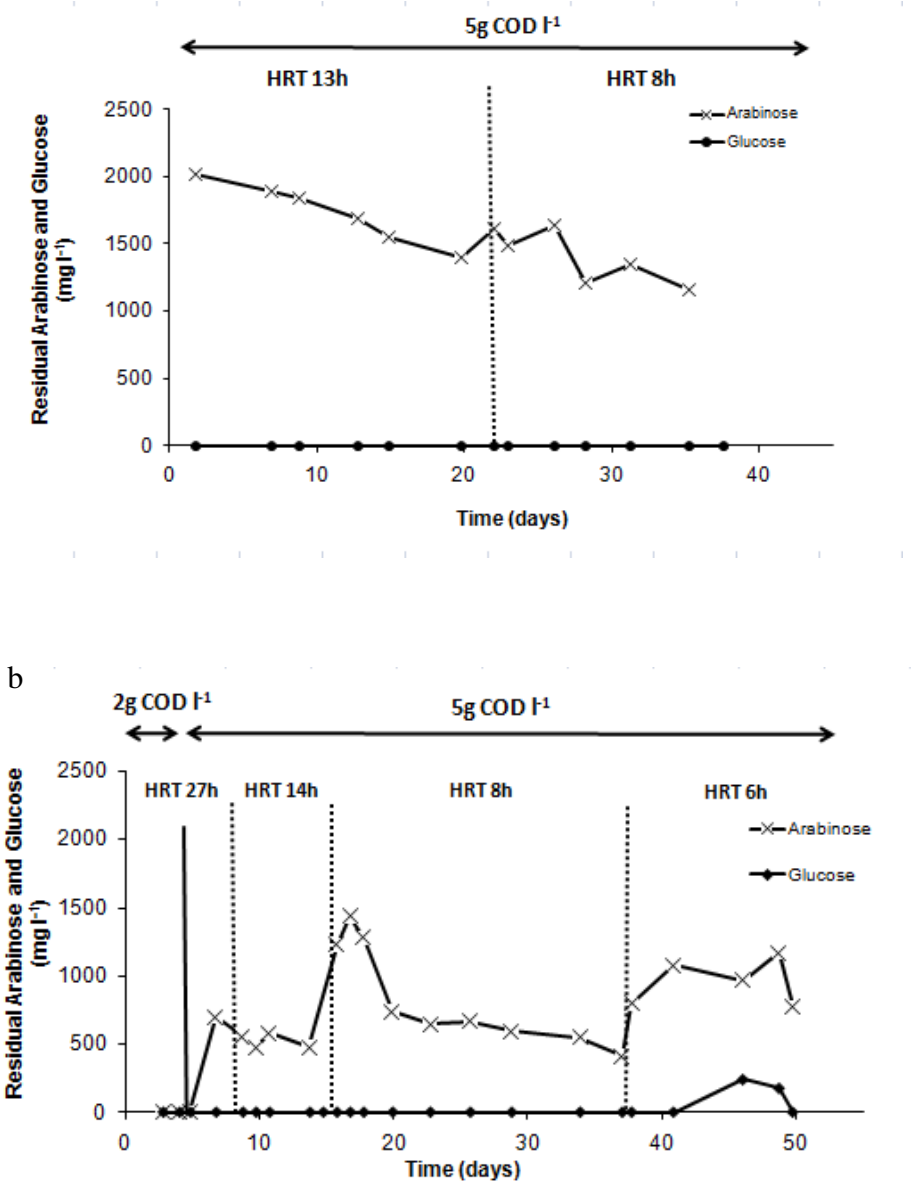

Figure 6.4. - Effect of HRT on arabinose and glucose utilization (a) R_{HTG} and (b) R_{EHTG}. Data represent average value from triplicate experiment, standard deviations were always within 5-10%.

Table 6.1. - Process performance of R_{EHTG} system and R_{HTG} system

Engineered heat treated granules system						Heat treated granules system					
Feed concentration (g COD l^{-1})	HRT (h)	Glucose utilization* (%)	Arabinose utilization* (%)	Conversion rate (mlH_2 g^{-1} substrate consumed)*	COD Balance (%)	Feed concentration (g COD l^{-1})	HRT (h)	Glucose utilization (%)	Arabinose utilization ** (%)	Conversion rate (mlH_2 g^{-1} substrate consumed)***	COD Balance (%)
2 (1arab+1gluc)	27	100	100	68±3	105						
5 (2.5arab+2.5gluc)	27	100	72	175±7	87±4	5 (2.5arab+2.5gluc)	13	100	31±8	50	90±5
	14	100	79±3	126±3	90±7		8	100	45±9	6	80±2
	8	100	74±5	130±8	109±0.3						
	6	94±5	60±7	95±1	101±2						

* Steady state average

** Average of all values from each HRT

*** No steady state achieved, the values correspond to peaks of conversation rate

All sugars were consumed in R_{EHTG} during the first days of operation, with the application of 2g COD L^{-1} total feed concentration. However, an increase of residual arabinose was observed in R_{EHTG} immediately after each decrease of HRT and when the total feed concentration was increased to 5g L^{-1} (Figure 6.4 b). The residual arabinose gradually decreased and stabilized around 600 mg L^{-1} for each HRT applied.

The present study indicates that glucose was preferentially consumed than arabinose when the feed concentration was higher than 2g COD L^{-1}. The increase of residual arabinose in R_{EHTG} immediately after lowering the HRT, suggest that the system needs some adaptation time to a new arabinose loading condition. This adaptation time was not observed for glucose, which was totally consumed after each decrease of HRT. Differences in simple sugars uptake can be explained with the different metabolic pathway of arabinose (pentose) vs glucose (hexose) catabolism (Hames and Hooper, 2000). Arabinose fermentation requires various enzymes, and consequently its biochemical reactions are relatively complex (Hames and Hooper, 2000). The complexity of metabolic pathways and the involvement of various enzymes can be one of the reasons for the transitory periods and preferential consumption of glucose. It is also known that, the presence of a rapidly biodegradable carbon (glucose) can inhibit the synthesis of enzymes involved in the metabolism of other carbon-containing compounds. The phenomenon is known as "the glucose effect" (Strobel, 1993) of repression of the other sugars catabolism.

Soluble fermentation products (SFP) profile

R_{HTG} showed a very unstable SFP profile with lactic acid as the most dominant fermentation product, apart from the periods of hydrogen production peaks where butyrate was the major SFP (Figure 6.3 c). Acetate and butyrate were the main fermentation products in R_{EHTG} during the application of HRT 27h and 14h (Figure 6.2 c). Thus, the bacterial metabolism in R_{EHTG} during these periods was likely following butyrate-acetate type fermentation, responsible for high

hydrogen yields in dark fermentation (Noike and Mizuno, 2000). However, other dominant SFP, i.e. lactate, became evident with the application of lower HRTs (8h and 6h). A butyrate-lactate fermentation type was probably occurring for the HRT of 8h. The application of HRT of 6h further induced a dominance of lactate and a decline of butyrate as fermentation products in R_{EHTG}. Propionate and ethanol were present in both reactors but the concentrations in most of the operation time were less than 250 mg L^{-1}. COD mass balance showed that the recovery of COD was high (80 to 109 %), indicating that experimental measurements were accurate (Table 6.1). The shift on the metabolic pathway from butyrate/acetate fermentation to lactate observed in R_{EHTG} was previously referred in other investigations on biohydrogen production (Liu et al., 2008). These authors indicated that lactic acid can be deemed as an indicator of organic overloading/inhibition for hydrogen fermentation process. Another study (Noike et al., 2002) reported the negative effect of lactic acid on continuous hydrogen production by a mixed culture; suggesting that the inhibition was caused by bacteriocins secreted by lactic acid bacteria. In the present study the substrate conversion rate to hydrogen was maintained with the HRT 8h, as well as the arabinose and glucose utilization, suggesting that the system was not overloaded during this period. Only with the application of HRT 6h the hydrogen yield decreased, suggesting that the system was probably overloaded. The increase of lactic acid could be also associated with the increase of hydrogen partial pressure already reported in other studies (Levin et al., 2004).

Microbial community profiles

Granules after heat treatment (HTG), used as R_{HTG} inoculum, and granules after the contact with the enriched culture (EHTG), used as R_{EHTG} inoculum, were analyzed in terms of bacterial community composition. The bacterial diversity established from the DGGE profiles, of each inoculum, was measured as Shannon diversity index (H). R_{HTG} inoculum presented lower Shannon diversity index (0.59) than R_{EHTG} inoculum (1.18), evidencing the importance of the contact of HTG with the enriched culture (E) for the increase of inoculum diversity. The surface of

HTG and of EHTG was also observed using SEM. The HTG exhibited an amorphous surface, while the presence of cocci- and bacillus–like cell type morphologies was observed in the granules surface of the EHTG (Figure 6.5). This highlights cellular integrity on the surface of EHTG, resulting most likely from the attachment of cells from the enriched culture.

Table 6.2. - Samples collected from R_{EHTG} and R_{HTG}, condition prevailing at the time of sampling

Sample	Operational Period	Feed concentration ($gCOD\ L^{-1}$)	Hydraulic retention time (h)
$R_{HTG}1$	R_{HTG} day 23	5	13
$R_{HTG}2$	R_{HTG} day 34	5	8
$R_{HTG}3$	R_{HTG} day 44	5	8
$R_{EHTG}1$	R_{EHTG} day 4	2	27
$R_{EHTG}2$	R_{EHTG} day 7	5	27
$R_{EHTG}3$	R_{EHTG} day 15	5	14
$R_{EHTG}4$	R_{EHTG} day 38	5	8

Comparison of the bacterial DGGE profiles obtained for EHTG and for the enriched culture (E) further supported the occurrence of attachment of specific microorganisms from the enriched culture in EHTG, as indicated by the presence of bands resolving at the same position in the gel (Figure 6.6). The specific bacterial community profiles in the granules of the two reactors had obviously been crucial for the different performance observed.

Along with the operation time, samples were taken from R_{HTG} and from R_{EHTG}, and their bacterial community composition was analyzed. Table 6.2 summarizes the condition prevailing at the time of sampling. The similarity indices (SI) between the DGGE profiles of each sample were compared. A high shift in the community composition compared to the original granules was observed during

the first period of operation in both R_{HTG} and R_{EHTG}, with similarity indices of 25 % and 3 %, respectively (Figure 6.6). Along operation time, the microbial community profile of R_{EHTG} went through a more pronounced change compared to bacterial community profiles of the R_{HTG}, as indicated from the lower similarity indices R_{EHTG} system ($R_{EHTG\ 1}/R_{EHTG2}$ - 36%, R_{EHTG2}/R_{EHTG3} - 42% and R_{EHTG3}/R_{EHTG4} - 53%) compared to corresponding profiles from HTG system (R_{HTG1}/R_{HTG2}-97 % and R_{HTG2}/R_{HTG3}-98 %).

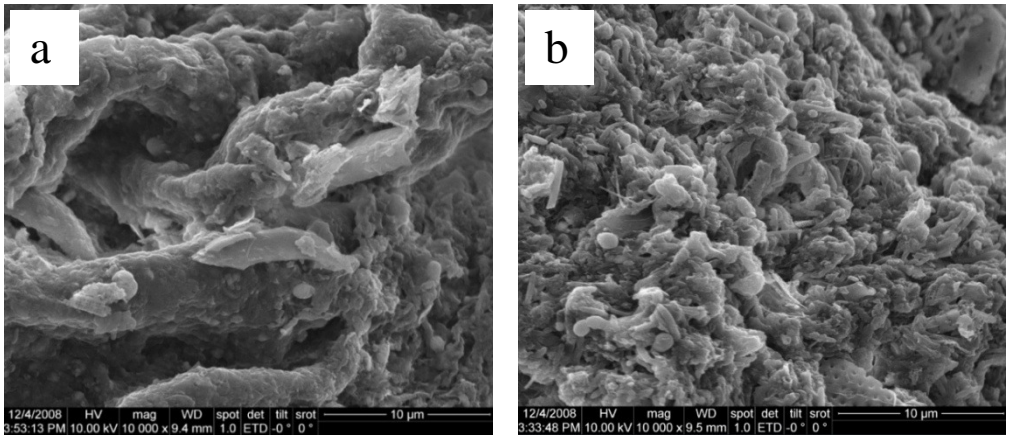

Figure 6.5. - SEM photographs of (a) surface of heat treated granules, (b) surface of granules after the contact with the enriched culture.

Further characterization of the bacterial community revealed that the dominant ribotypes in the enriched culture were most closely affiliated to *Thermoanaerobacter*-like organisms (99-100% identity) and *Caldicoprobacter*-like organisms (99%) (Table 6.3). Besides this major genus, clone inserts with sequences similar to those of uncultured bacterium related to *Clostridia* class was also retrieved from the enriched culture. The clone corresponding to band10 showed 99% identity to an uncultured bacterium found in a hydrogen-producing extreme thermophilic anaerobic microflora enriched from cow manure and xylose (Yokoyama et al., 2007).

In R_{EHTG} system, clone inserts closely related to genera *Clostridium*, *Sporolactobacillus*, *Bacillus*, *Klebsiella* and *Thermoanaerobacterium* were

obtained (Table 6.3). Clones exhibiting high sequence identity to *Clostridium beijerinckii* (99% identity), *Clostridium corinoforum* (99%) and *Klebsiella* sp. (99%) (Figure 6.6 and Table 6.3) corresponded to bands resolving in the upper positions of DGGE profiles from R_{EHTG} (bands 1, 2 and 3) but were apparently absent in R_{HTG}.

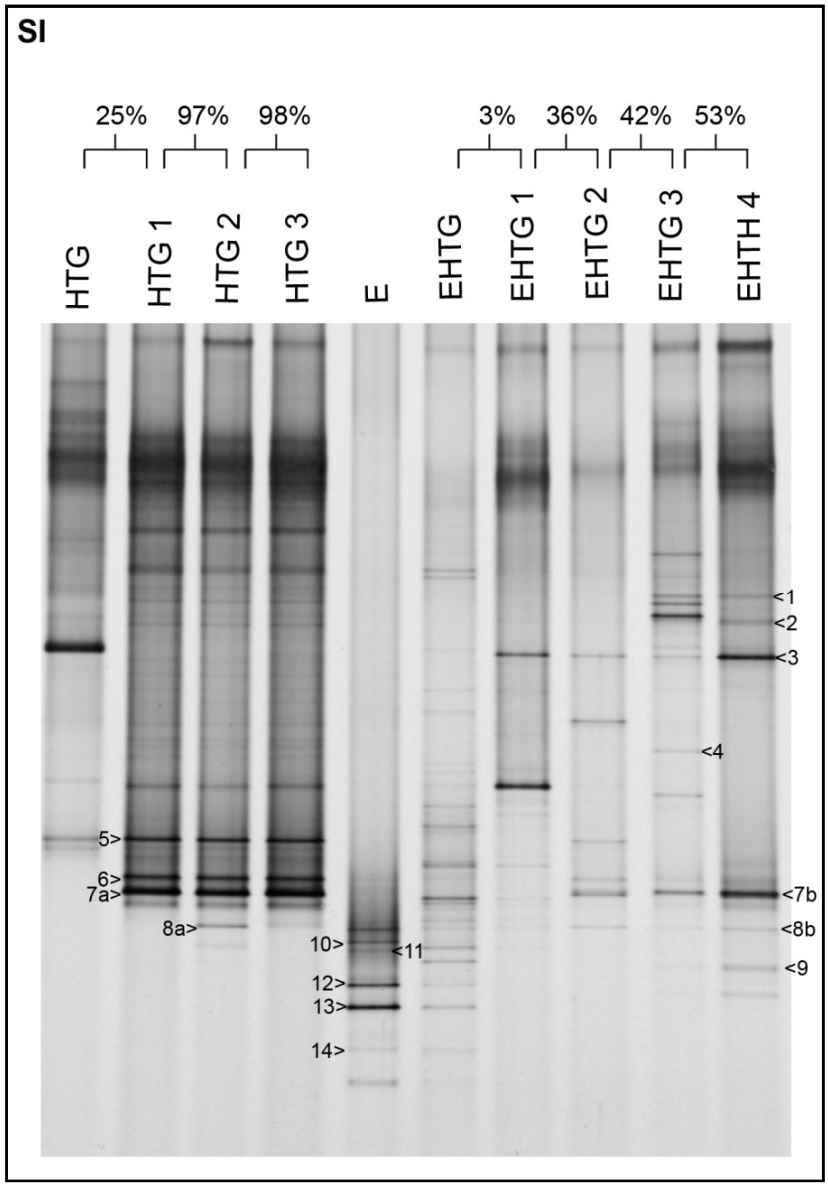

Figure 6.6. - DGGE profile of granules before (G) and after heat treatment (HTG), the enriched culture (E) and granules after the contact with the enriched culture (EHTG); heat treated granules system on day 23 (HTG1), day 34 (HTG2) and day 44 (HTG3); engineered heat treated granules system on day 4 (EHTG1), day 7 (EHTG2), day 15 (EHTG3) and day 38 (EHTG4). Similarity index (SI).

The presence of those potential hydrogen producing bacteria clustering within the *Clostridium* and *Klebsiella* genus, was the main difference found when comparing the DGGE profiles (number and intensity of bands) of both systems. Previous studies have also reported the potential of using *Clostridium* and *Klebsiella* to produce hydrogen from a variety of carbohydrates (Sen Gupta et al., 2005).

Bacillus-like organisms were also found in R_{EHTG} DGGE profiles, and during the transient peaks of hydrogen production in R_{HTG} (band 8a and 8b in lanes HTG2 and EHTG4 in Table 6.4 and Figure 6.6). Microorganisms affiliated with the *Thermoanaerobacterium* genus, were present in both, R_{HTG} and R_{EHTG} DGGE profiles (Table 6.3 and Table 6.4). However, in R_{EHTG} the band corresponding to a closest relative of *Thermoanaerobacterium thermosacharolyticum* (band 7b) became stronger only at the end of operation time*. T. thermosacharolyticum* is known by the capability of producing hydrogen as well as lactate and volatile fatty acids from a variety of sugars, including glucose and arabinose (Altaras et al., 2001). *T. thermosacharolyticum* was also immobilized on heat treated methanogenic granules by O-Tong and co-authors for high-rate continuous hydrogen production (Thong et al., 2008).

The presence of an enriched and active hydrogen producing culture during the start-up of R_{EHTG} system might be crucial for the efficient hydrogen production afterwards, since lactic acid was not produced in high concentration in R_{EHTG} during the first periods of operation as it was in R_{HTG} system. Organisms from class *Clostridia* (closest related to *Thermoanaerobacter subteraneus*, *Thermoanaerobacter tengcongensis* and *Caldicoprobacter oshimai*) and uncultured bacteria; (lane E, Figure 6.6) were present initially in the enriched hydrogen producing culture but not *Thermoanaerobacterium thermosacharolyticum.* After the batch period, the R_{EHTG} was fed continuously. After 4 and 7 days of continuous operation, a low microbial diversity was observed and bands corresponding to organisms present in the enriched culture were not detected (Figure 6.6, lanes EHTG1, EHTG2, respectively).

Table 6.3. - Phylogenetic affiliations of cloned 16SrRNA gene sequences corresponding to identified bands in the DGGE profiles

BM[a]	Phylum[b]	Class[b]	Affiliation[c]	Acc[d]	Identity (%)
1	*Firmicutes*	*Clostridia*	*Clostridium corinoforum* (X76742.1)	GU296463	99
2	*Firmicutes*	*Clostridia*	*Clostridium beijerinckii* (EF446166.1)	GU296464	99
3	*Proteobacteria*	*Gammaproteobacteria*	*Klebsiella* sp (DQ358738.1)	GU296465	99
4	*Firmicutes*	*Bacilli*	*Sporolactobacillus inulinos* (AB362770.1)	GU296466	96
5	*Firmicutes*	*Clostridia*	*Thermoanaerobacterium thermosacharolyticum* (EF680277.1)	GU296467	97
6	*Firmicutes*	*Clostridia*	*Thermoanaerobacterium thermosacharolyticum* (EU563362.1)	GU296468	99
7a	*Firmicutes*	*Clostridia*	*Thermoanaerobacterium thermosacharolyticum* (EF680277.1)	GU296469	99
7b	*Firmicutes*	*Clostridia*	*Thermoanaerobacterium thermosacharolyticum* (EF680277.1)	GU296470	100
8a	*Firmicutes*	*Bacilli*	*Bacillus coagulans* (DQ297928.1)	GU296471	99
8b	*Firmicutes*	*Bacilli*	*Bacillus coagulans* (AB116136.1)	GU296472	99
9	*Firmicutes*	*Bacilli*	*Sporolactobacillus nakayamae* (AB362637.1)	GU296473	95
10	*Firmicutes*	*Clostridia*	Uncultured bacterium (AB286973.1)	GU296474	99
11	*Firmicutes*	*Clostridia*	*Caldicoprobacter oshimai* (AB450762.1)	GU296475	99
12	*Firmicutes*	*Clostridia*	*Thermoanaerobacter subterraneus* (AY216597.1)	GU296476	99
13	*Firmicutes*	*Clostridia*	*Thermoanaerobacter subterraneus* (AY216597.1)	GU296477	99
14	*Firmicutes*	*Clostridia*	*Thermoanaerobacter tengcongensis* (AE008691.1)	GU296478	99

[a]Band mark in Figure 6.6

[b]Classified using the RDP Naive Bayesian Classifier (Wang et al., 2007)

[c]Closest organisms in GenBank with accession number

[d]GenBank accession number

In this period, hydrogen yield was maximal in this reactor (Table 6.1). Also the profile of microbial fermentation products (SFP) revealed that acetate was the main compound detected in the R_{EHTG} effluent. Following these initial days, the hydrogen production in this reactor was stable, although the hydrogen yields tended to decrease slightly. This finding was associated with a change in the profile of soluble fermentation products where lactate increased, especially after day 38, likely due to the decrease in the hydraulic retention time. The interesting point is that this operational behavior of R_{EHTG} was associated to the increasing intensity of a band affiliated with *T. thermosacharolyticum* (100% 16S rRNA gene sequence identity).

Table 6.4. - Samples collected from R_{EHTG} and R_{HTG}, reactors performance and dominant bacteria ribotypes present

Sample	Process Performance ($mLH_2\ L^{-1}\ d^{-1}$)	Closest organisms in GenBank
$R_{HTG}1$	2	*Thermoanaerobacterium thermosacharolyticum*
$R_{HTG}2$	1047	*Thermoanaerobacterium thermosacharolyticum; Bacillus coagulans*
$R_{HTG}3$	56	*Thermoanaerobacterium thermosacharolyticum*
$R_{EHTG}1$	147	*Klebsiella*sp
$R_{EHTG}2$	975	*Klebsiella* sp.*; Thermoanaerobacterium thermosacharolyticum; Bacillus coagulans*
$R_{EHTG}3$	1380	*Clostridium corinoform; Klebsiella* sp; *Sporolactobacillus inulinus; Thermoanaerobacterium thermosacharolyticum; Bacillus coagulans*
$R_{EHTG}4$	2597	*Clostridium corinoform; Clostridium beijerinckii; Klebsiella* sp*; Thermoanaerobacterium thermosacharolyticum; Bacillus coagulans; Sporolactobacillus sp.*

Increasing lactate production by *T. thermosacharolyticum* was reported before by Vancanneyt (1990) at increasing dilutions rates, which may explain the partial shift from hydrogen to lactate, observed in the experiment at the lower hydraulic retention times. Notwithstanding, the hydrogen production was still significant in later operational periods, likely due to the presence of organisms closely related to class *Clostridia* (*Clostridium beijerinckii, Clostridium corinoforum)* and class *Gammaproteobacteria* (*Klebsiella* sp.) (Table 6.4, Figure 6.2).

The global history of R_{HTG} clearly indicates that hydrogen was only occasionally produced when lactate was minimal and that *T. thermosacharolyticum* -related bacteria were dominant in the operational time, although a band corresponding to a closest relative to *Bacillus coagulans* was detected in day 34.

It is therefore clear that the contact of the heat treated granules with the enriched culture favored the continuous hydrogen production. However, immobilization of specific microorganisms of the enriched culture, such as *Thermoanaerobacter subterraneus*, *Thermoanaerobacter tengcongensis* and *Caldicoprobacter oshimai,* apparently did not occur, suggesting that the microbial advantage of the EHTG reactor by comparison with the HTG was not direct and therefore the explanation is not straightforward. Some hypotheses can be pointed out:

1) In the R_{EHTG}, the presence, since the beginning, of an active hydrogen producing community promoted a different SFP profile, more favorable to hydrogen production. That likely resulted from the competition between the culture and the glucose and arabinose consumers present in the heat treated granules, which were still under activation. Interestingly, dominant organisms in the enriched culture, *Thermoanaerobacter subterraneus* (99% 16S rRNA gene sequence similarity) and *Thermoanaerobacter tengcongensis* (99%), might not consume arabinose (Fardeau et al., 2000; Xue et al., 2001). Therefore, arabinose was likely available for other organisms eventually present in the culture but not dominant in the beginning. In particular, *Klebsiella* sp. is referred as a promising organism capable of utilizing pentose as carbon source to

produce hydrogen (Rosenberg, 1980). From Figure 6.6, band 3, the presence of *Klebsiella* –like organisms is evident during reactor operation. The different SFP profile could have selected for a microbial community able to produce hydrogen in this reactor.

2) Another possible explanation for the different behavior observed in the two reactors can be the antibiotic influence of some clostridial species (ribotypes corresponding to bands 10-14, lane E, Figure 6.6) present in the enriched culture, which could have favored, in R_{EHTG}, the selection of hydrogen producing organisms in detriment of lactate producing ones. Although antibiotic production from thermophilic *Clostridia* has not been reported so far, antibiotic potential of other thermophiles such as bacilli and actinomycetes including was already described (Edwards, 1993; Esikova et al., 2002).

6.4 Conclusions

Significant improvement of hydrogen production and process stability was obtained in engineered heat treated granular system compared with heat treated granular system. The presence of potential hydrogen producing bacteria clustering within the *Clostridium* and *Klebsiella* genera was the main difference found between both systems, which is likely to be related to the better performance exhibited by the R_{EHTG} system. The contact of HTG with an enriched active hydrogen producing culture during the reactor start-up contributed to the development of a stable and efficient hydrogen production during the R_{EHTG} system operation.

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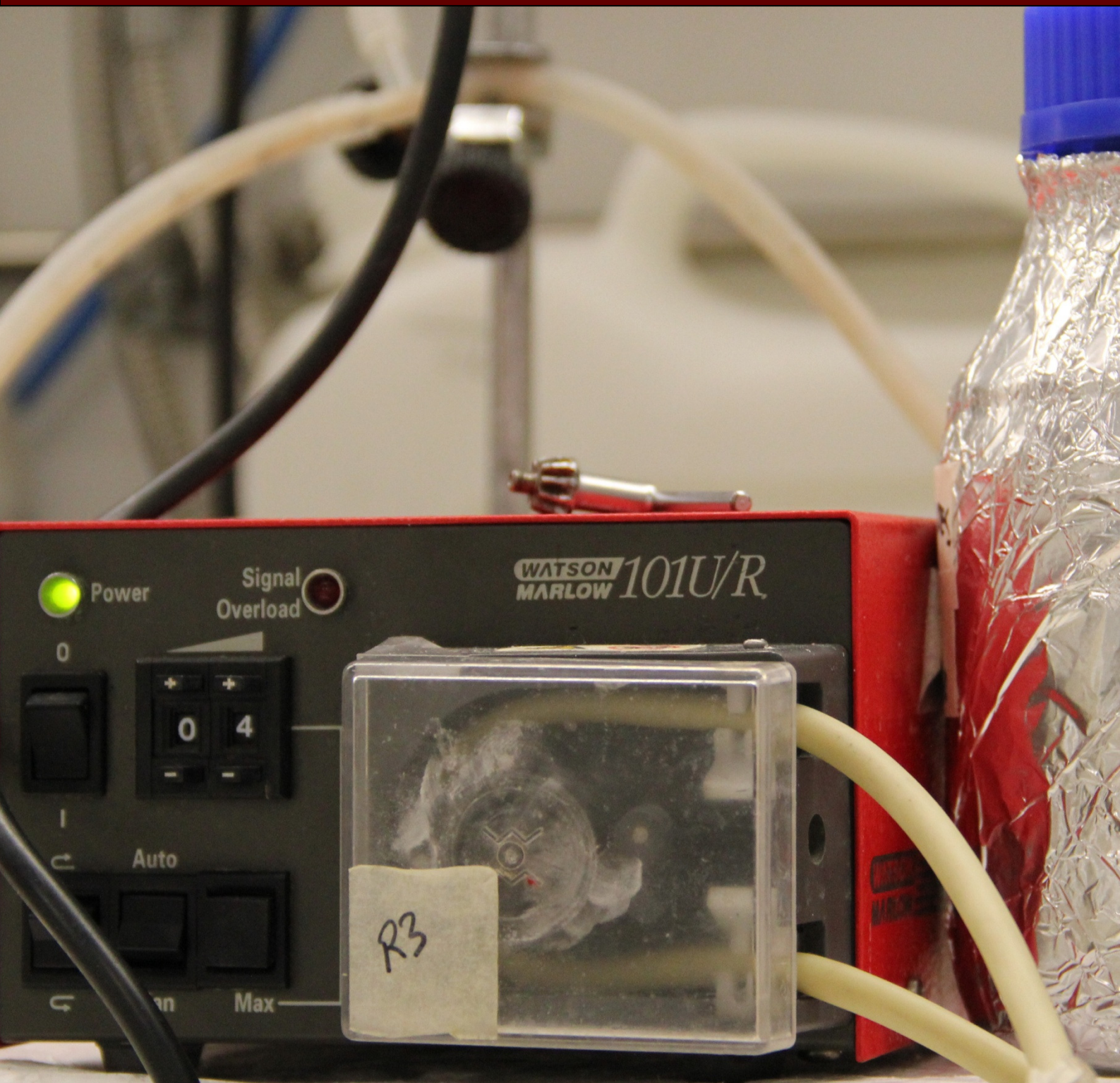
Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures

Chapter 7



Abstract

Conversion of arabinose (C5 sugar) and glucose (C6 sugar) to hydrogen, by extreme thermophilic anaerobic mixed cultures was studied in continuous ($70^{o}C$, pH 5.5) and batch ($70^{o}C$, pH 5.5 and pH 7) mode. Two EGSB reactors, R_{arab} and R_{gluc}, were continuously fed with arabinose and glucose, respectively. No significant differences on the reactors' performance were observed for low feed sugar-concentrations. However for higher sugar loads, R_{arab} showed better overall performance with higher hydrogen production rate (i.e. 3.2 $LH_2\ L^{-1}\ d^{-1}$) and yield (1.10 $molH_2$ per mole of substrate consumed) than the ones measured in R_{gluc} (2.0 $LH_2\ L^{-1}\ d^{-1}$ and 0.75 $molH_2$ per mol of substrate consumed). Lower hydrogen production in R_{gluc} was associated to a higher lactate production. DGGE results revealed no significant difference on the bacterial community composition between operational periods and reactors, suggesting that the higher concentration of lactate was not a consequence of bacterial community shift, but resulted from a change on the main metabolic pathways of glucose catabolism. Hydrogen partial pressure and pH could also be linked to efficiency in hydrogen production, using batch assays. An increase of hydrogen production from both substrates was observed when hydrogen partial pressure is kept low. Sugars uptake was enhanced and hydrogen production stimulated when pH 7 was used.

7.1 Introduction

Hydrogen is considered a promising renewable energy carrier that can contribute towards a low carbon economy. Fermentative hydrogen production from carbohydrate-containing feedstock, such as glucose, sucrose and starch, has been extensively studied (Arooj et al., 2008; Lee et al., 2008; Zheng et al., 2008). However, second generation hydrogen fermentation technologies, using organic agricultural and forestry wastes, are presently emerging as promising and more cost-effective solutions (Levin et al., 2004; Shin et al., 2004). Besides, the utilization of wastes in dark fermentative hydrogen processes merges treatment technology and clean energy production (Kapdan and Kargi, 2006).

Lignocellulosic material, which is the main component of agricultural and forestry wastes, must be pre-treated prior to fermentation to hydrogen in order to remove lignin and hemicelluloses and to decrease cellulose crystallinity. Physico-chemical pre-treatment of lignocellulosic material, such as hydrothermal treatment or wet oxidation, yields a two phase product, i.e. a solid phase mainly consisting of hexoses (e.g. glucose), and a hydrolysate mainly containing pentoses (e.g. L-arabinose and D-xylose) (Thomsen et al., 2006). Efficient microbial fermentation of hexoses and pentoses is, therefore, the key step for hydrogen production from plant biomass. However, combined fermentation of mixtures of hexoses and pentoses is often prevented due to catabolite repression; in the presence of glucose, pentoses might be converted to a lesser extent thereby decreasing overall fermentation yields. Moreover, efficient hydrogen production from sugars is dependent on the different possible fermentation pathways. Most of the extreme thermophiles from the phylum *Clostridia* use the Embden-Meyerhof pathway to metabolize hexose sugars to pyruvate. Biohydrogen can be then formed via decarboxylation of pyruvate to acetyl CoA, in which reduced ferredoxin (Fd_{red}) is generated and acts as a direct electron donor for proton reduction to hydrogen (Figure 2.2). Maximum hydrogen yield, both from hexoses or pentoses, is obtained with acetate as the fermentation product from glucose (equations 1 and 2). Low yields are

associated to the formation of butyrate, propionate and reduced end products, such as alcohols and lactic acid.

$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COO^- + 2CO_2 + 2H^+ + 4H_2$ (1)

$C_5H_{10}O_5 + 1.67H_2O \longrightarrow 1.67CH_3COO^- + 1.67CO_2 + 1.67H^+ + 3.33H_2$ (2)

Operating fermentation processes at thermophilic (45-60^oC) and extreme thermophilic (65-80^oC) conditions has been shown to favor the formation of acetate, thereby yielding more hydrogen (Kadar et al., 2004). High temperatures inhibit the proliferation of methanogenic archaea and homoacetogenic bacteria; this is an important advantage when using mixed-cultures for hydrogen production because it prevents hydrogen from being consumed by these groups before being recovered from the system (as is often the case in mesophilic fermentations) (van Groenestijn et al., 2002). Also, higher rates of hydrolysis of cellulosic material have been observed in studies performed at thermophilic conditions, with the concurrent formation of higher amounts of fermentable sugars (Lu et al., 2008). Presently, there are a considerable number of studies on H_2 production at extreme thermophilic conditions using pure culture fermentation (van Niel et al., 2002; van Niel et al., 2003), but only few studies using mixed-cultures (Kongjan et al., 2010). Hydrogen production by mixed culture fermentation is more suited for industrial applications, when compared to pure culture fermentation. Some of the advantages are: (i) no need for sterile cultivation, (ii) presence of high microbial diversity, which offers increased adaptation capacity, (iii) possibility of mixed substrates co-fermentation, and (iv) higher capacity for continuous processing (Kleerebezem and van Loosdrecht, 2007; Temudo et al., 2007).

In the present study, continuous expanded granular sludge bed (EGSB) reactors were used to study the conversion of a C5-sugar (arabinose) and a C6-sugar (glucose) to hydrogen, using anaerobic mixed-cultures under extreme thermophilic conditions (70^oC). Microbial diversity present in arabinose- and glucose-fed bioreactors was assessed using a PCR-DGGE approach. Additional batch experiments were performed to study the effect of hydrogen partial pressure and pH on the hydrogen production from arabinose and glucose.

7.2 Materials and methods

Continuous hydrogen production in EGSB reactors

Experiments were carried out in two plexi-glass expanded granular sludge reactors (EGSB), reactor arabinose (R_{arab}) and reactor glucose (R_{gluc}) fed with L-arabinose and glucose, respectively. EGSB reactors had a height of 1.95 m and internal diameter of 21 mm. Total liquid volume was 1.30 L, including reaction-zone volume of 0.7 L. Reactors were operated at 70 ± 1 ^{o}C by means of an external water jacket, and pH inside the reactors was maintained at 5.5±0.5. Superficial velocity was set at 10.0 m h^{-1} (using internal recirculation) with an hydraulic retention time (HRT) of 9 h. Before start-up, R_{arab} and R_{gluc} were inoculated with 400 mL of granular sludge from a lab-scale hydrogen-producing reactor that had been fed with a mixture of arabinose (17 mM) and glucose (14 mM) for 2 months. Start-up of R_{arab} was done using a constant arabinose feed concentration of 10.0 mM (period I); afterwards, arabinose concentrations of 16.6 mM (period II) and 33.3 mM (period III) were tested. Start-up of R_{gluc} was done using a constant glucose feed concentration of 8.3 mM (period I); afterwards, concentrations of 13.8 mM (period II) and 27.7 mM (period III) were tested. The concentrations of arabinose and glucose used differed in order to have substrate concentrations that theoretically yield the same amount of hydrogen (i.e. 33.3, 55.5 and 110.8 mM H_2 for periods I, II and III, respectively). Sodium bicarbonate was added to the feed as alkalinity source (at a final concentration of 1 to 2 g L^{-1}). Macronutrients (0.6 mL g^{-1}COD) ($MgSO_4.7H_2O$: 30 g L^{-1}; KH_2PO_4: 28.3 g L^{-1}; NH_4Cl: 170 g L^{-1}) necessary for cell growth were also added.

Batch experiments

Seed sludge - Granular sludge used for inoculating batch assays for studying arabinose- and glucose-conversion was collected from reactors R_{arab} and R_{gluc}, respectively.

Medium composition and substrates – A 20 mM phosphate-buffered medium was used for experiments at pH 5.5, while for experiments at pH 7 a bicarbonate-buffered medium equilibrated with a gas phase of 80% N_2 and 20% CO_2 was used. Both media were supplemented with trace elements, salts and vitamins according to the procedure described by (Stams et al., 1993). Assays were performed using 18 mL of medium in 70 mL serum bottles containing 0.4 g of granular sludge. Bottles were sealed and flushed with N_2 or N_2/CO_2 mixture for pH 5.5 and pH 7 assays, respectively. Medium was subsequently reduced with 0.8 mM sodium sulfide ($Na_2S.9H_2O$). Yeast extract was added to the medium to a final concentration of 0.5 g L^{-1}. L-arabinose and glucose were individually used as substrate and added to the medium to final concentration of 13 mM and 11 mM, respectively. Substrates were supplemented at the beginning of the assay and, after depletion of the substrate, a second pulse of 13 mM arabinose or 11 mM glucose was added to the medium. All the incubations were performed at 70^{o}C without shaking and in the dark.

Effect of hydrogen partial pressure - The effect of hydrogen partial pressure on hydrogen production from arabinose and glucose was investigated in batch mode at pH 5.5. Two sets of batch experiments were performed: in one set, hydrogen was allowed to accumulate in the gas phase – NHR (no headspace replacement) assays; in the other set, formed hydrogen was removed from the bottles headspace and gas volume replaced by 100% N_2 – HR (headspace replacement) assays. All the experiments were performed in triplicate. Blanks without substrate were also performed. Sugars and soluble fermentation products (SFP) concentration in the medium and hydrogen in the gas phase were measured during batch fermentations. Dissolved hydrogen concentration was calculated using the Henry's law at 70ºC: K_H*Pi, where K_H is the Henry's law constant for hydrogen (8.7x10^{-9} M/Pa @ 70^{o}C).

Effect of pH - The effect of pH on hydrogen production and soluble microbial products produced from L-arabinose and glucose was studied in batch mode at

70ºC. Two sets of batch experiments were performed, one at pH 7 and the other at pH 5.5. All the experiments were done in triplicate. Sugar and SFP concentration in the medium and hydrogen in the gas phase were measured during batch fermentations. Dissolved hydrogen concentration was also calculated using the Henry's law at 70^{o}C.

Analytical methods

Hydrogen in the gas phase was determined by gas chromatography (GC) using a Hayesep Q column (80/100 mesh) and thermal conductivity detector (Varian 3300 Gas Chromoatograph) with nitrogen (30 mL min^{-1}) as the carrier gas. The injector, detector, and column temperatures were 120, 170, and 35ºC respectively. Methane and carbon dioxide content of the gas phase from batch experiments and EGSB reactors was determined by gas chromatography using a *Porapack Q* (100 - 180 mesh) column, with helium as the carrier gas at 30 mL min^{-1}, and a thermal conductivity detector. Temperatures of the detector, injector and oven were 110, 110 and 35ºC, respectively. In the EGSB reactors gas flow rate was measured by a *Ritter Milligascounter* (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany). Volatile fatty acids (VFA), ethanol, lactic acid, L-arabinose, and glucose were determined by high performance liquid chromatography using an HPLC (Jasco, Japan) with a *Chrompack column* (6.5 x 30 mm^{2}); sulfuric acid (0.01 N) at a flow rate of 0.7mL min^{-1} was used as mobile phase. Column temperature was set at 60 ºC. Detection of VFA, lactic acid, ethanol, arabinose, glucose was made sequentially using a UV detector at 210nm and a RI detector.

PCR -DGGE

Representative granular sludge samples were collected from R_{arab} and R_{gluc} and stored at -18ºC. Total genomic DNA was extracted from approximately 500 µL of sample by using a FastDNA SPIN kit for soil (Qbiogene, Carlsbad, CA, USA). 16S rRNA gene fragments of approximately 450 bp were amplified for DGGE analysis

by PCR using a Taq DNA polymerase kit (Life Technologies, Gaithersburg, MD, USA) using the primer set 954GC-f and 1369-r, as previously described by (Nubel et al., 1996). The size of the obtained PCR products was checked by comparison with appropriate size and mass standard (MBI Fermentas, Vilnius, Lithuania), by electrophoresis on an 1% (w/v) agarose gel and ethidium bromide staining. Gels ran at a constant voltage of 100 V in an agarose gel electrophoresis system (Mupid-EX, Belgium). Nucleic acids were detected using an UV transilluminator (BioRad).

DGGE analysis of the amplicons was done by using the DCode system (Bio-Rad, Hercules, CA, USA). PCR products were electrophoresed in a 0.5x Trisacetate-EDTA buffer for 16h at 85V and 60ºC on polyacrylamide gel (8%) containing a linear gradient ranging from 30% to 60% denaturant. Silver staining of DGGE gels was performed as previously described (Sanguinetti et al., 1994). DGGE gels were scanned at 400 dpi and the DGGE profiles compared using the Bionumerics 5.0 software package (Applied Maths, Belgium). Similarity indices (Si) of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product-moment correlation (Hane et al., 1993). Community shifts were described as changes in the DGGE profiles of the partial 16S rDNA amplicons.

Gibb's Free energy calculations

Standard Gibb's free energy at 25ºC (ΔG^{O}) was calculated using standard Gibb's free energy of formation values (ΔG^{O}_{f}) obtained in literature (Mavrovouniotis, 1991; Thauer et al., 1977).

7.3 Results

EGSB reactors performance

Hydrogen production rates in the EGSB reactors fed with arabinose (R_{arab}) and with glucose (R_{gluc}) are shown in Figure 7.1 Figure 7.2, respectively. During start-

up (period I), hydrogen production rates of approximately 0.3 LH_2 $L^{-1}d^{-1}$ were observed in both R_{arab} and R_{gluc}. This corresponds to a hydrogen yield of roughly 0.2 and 0.3 mol per mol of substrate consumed, for R_{arab} and R_{gluc} respectively (Table 7.1).

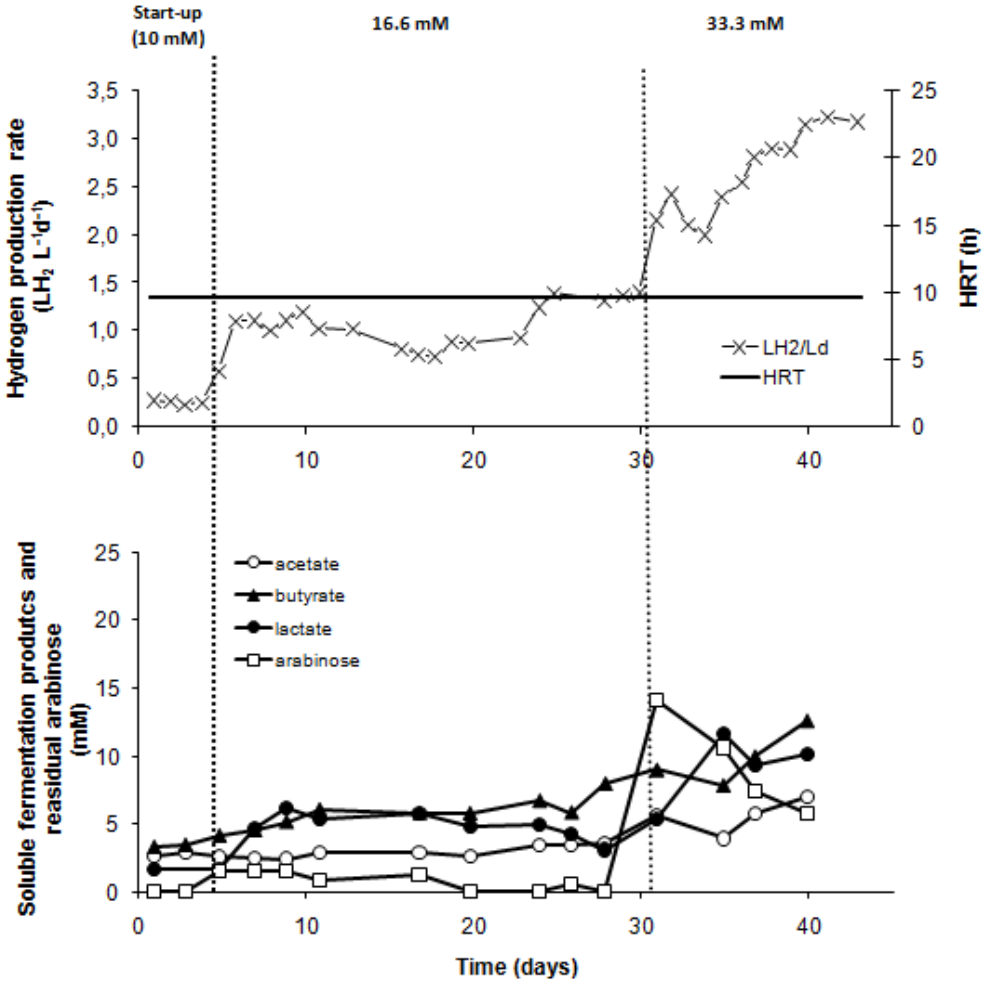


Figure 7.1. - Effect of OLR on performance of R_{arab} (a) hydrogen production rate and HRT, (b) soluble fermentation products and residual arabinose. Data represent average value from triplicate experiment, standard deviations were always within 5-10%.

In period II, the increase in arabinose and glucose inlet concentrations to 16.6 mM and 13.8 mM, respectively, resulted in hydrogen yields of about 0.8 mol per mole of substrate in both R_{arab} and R_{gluc}. Maximum hydrogen production rates in period II were of 1.36±0.04 and 1.12±0.07 LH_2 L^{-1} d^{-1} in R_{arab} and R_{gluc}, respectively. Substrate was completely consumed in both reactors and by-products formed were mainly butyrate, acetate and lactate (Figure 7.1Figure

7.2). Butyrate was the most abundant by-product formed in R_{arab} and R_{gluc} with average concentrations above 5 mM; concentration of lactate in both reactors was always higher than acetate. In operation period III, substrate concentrations fed to R_{arab} and R_{gluc} were increased to 33.3 mM of arabinose and 27.7 mM of glucose, respectively. Hydrogen production rate in steady state in R_{arab} was 3.26 ±0.16 $LH_2\ L^{-1}\ d^{-1}$ and in R_{gluc} was 2.06±0.06 $LH_2\ L^{-1}\ d^{-1}$.

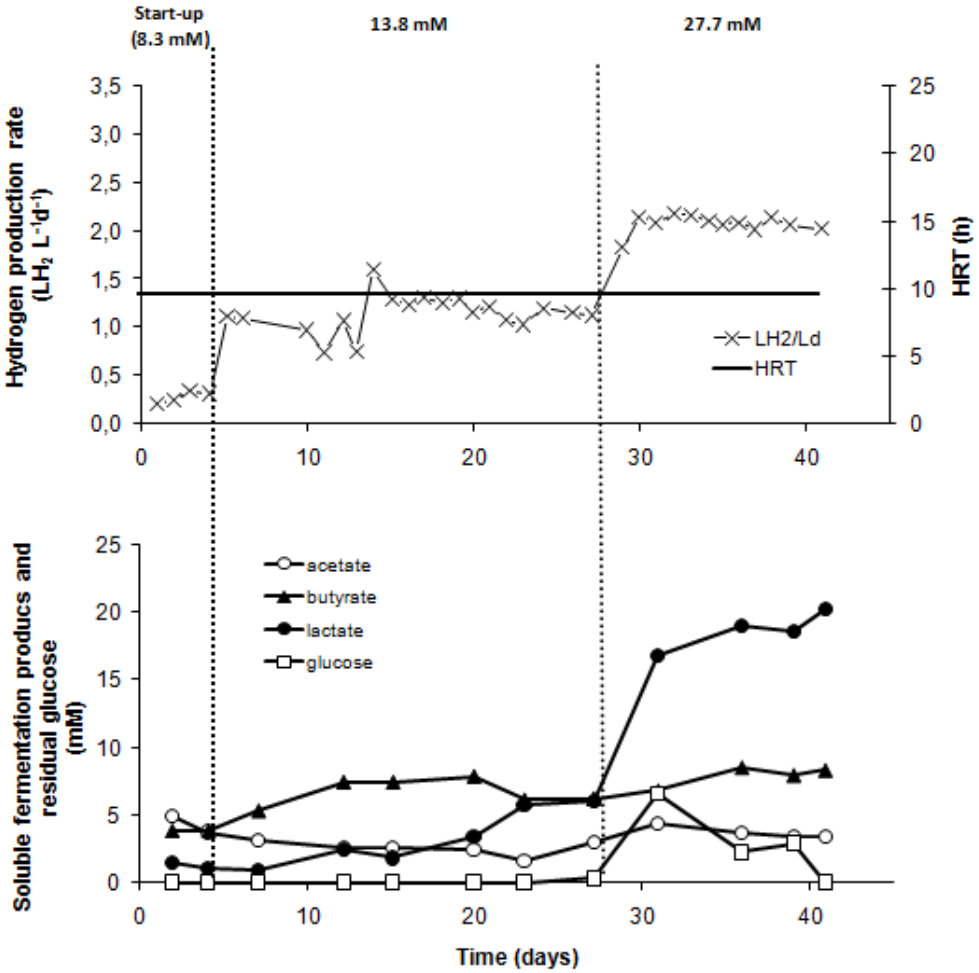


Figure 7.2. - Effect of OLR on performance of R_{gluc} (a) hydrogen production rate and HRT, (b) soluble fermentation products and residual glucose. Data represent average value from triplicate experiment, standard deviations were always within 5-10%.

Substrate was not completely consumed at the beginning of period III but, after 13 days of acclimation to the higher substrate loads virtually all glucose and average 79% arabinose were used (Table 7.1). During period III hydrogen yield in

R_{gluc} was stable and about 0.75 $molH_2$ per mole of substrate consumed. Hydrogen yield in R_{arab} was higher, i.e. 1.10 $molH_2$ per mole of substrate consumed. In period III, lactate became the dominant by-product in R_{gluc}, attaining a concentration of approximately 20 mM (Figure 7.2). In R_{arab}, lactate concentration also increased during period III, but did not exceed 11 mM (Figure 7.1). Butyrate and acetate were present in both R_{arab} and R_{gluc}, but at higher concentrations in R_{arab}. Propionate and ethanol were detected in R_{arab} and Rgluc but always at concentrations lower than 1 mM. No methane was ever detected in gas phase of R_{arab} and R_{gluc}.

Bacterial community composition dynamics in EGSB reactors

DGGE profiles generated for sludge samples withdrawn from R_{arab} and R_{gluc} show that bacterial composition in both reactors sludge at the end of periods II (day 27) and III (day 41) are identical (Figure 7.3). Similarity indices of Arab/Gluc samples at days 27 and 41 were as high as 88% and 94%, respectively. Predominant DGGE bands present in R_{gluc} and R_{arab} were also present in the inoculum used in this study for which the phylogeny has been previously assessed by cloning and sequencing (Abreu et al., 2010). Two of the predominant DGGE bands correspond to bacteria highly similar (>99%) to the hydrogen-producing *Thermoanaerobacterium thermosaccharolyticum*. Members of the *Klebsiella*, *Bacillus* and *Sporolactobacillus* genera, detected in the inoculums sludge, are also predominant in R_{gluc} and R_{arab}.

Effect of hydrogen partial pressure and pH on batch hydrogen production from arabinose and glucose

Two important parameters influencing hydrogen production were studied: partial pressure of hydrogen and pH of the liquid phase. The effect of the hydrogen partial pressure, both from arabinose and from glucose, was studied in batch experiments at pH 5.5 (equivalent to 5.0@70^{0}C). The effect of using a higher pH (i.e. pH 7, 6.5 @70^{0}C) was further studied. Assays were performed

allowing accumulation of hydrogen in the headspace (no headspace removal assays, NHR), or preventing hydrogen accumulation in the headspace (headspace removal assays, HR).

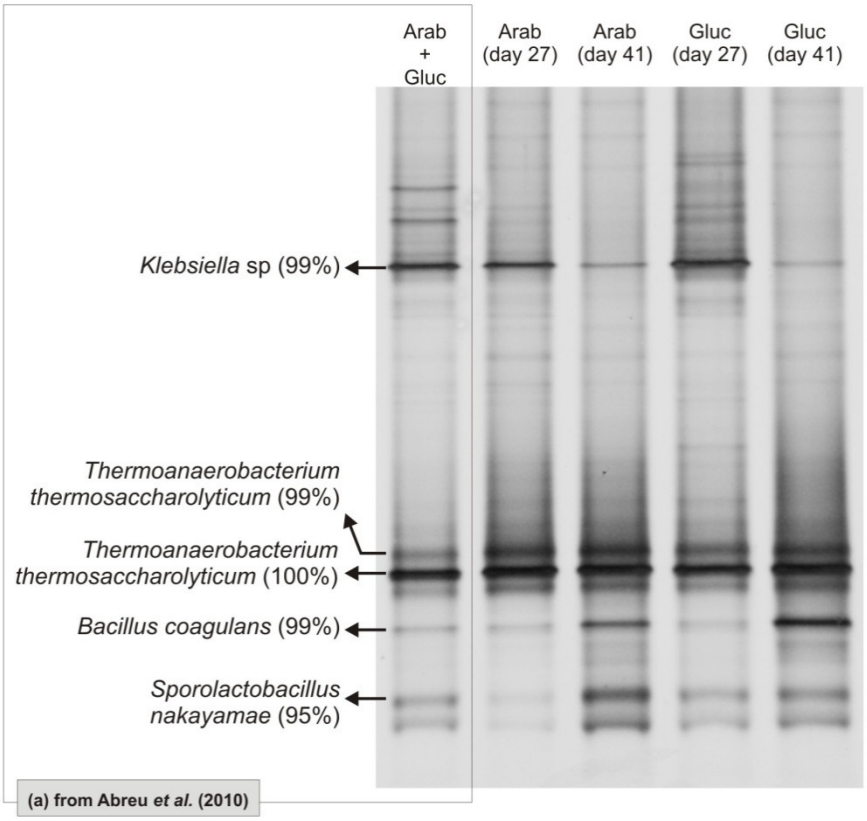


Figure 7.3. – DGGE profile of granular sludge samples collected from a reactor fed with a mixture of arabinose and glucose (arab + glu) (Abreu et al., 2010) and at day 27 and day 41 from arabinose (Arab1, Arab2) and glucose (Gluc1, Gluc2) reactors.

In the NHR arabinose and glucose assays at pH 5.5, the maximum hydrogen concentration in the gas phase was achieved after 44 and 20 h of the second substrate addition, respectively (Figure 7.4 a, b). At this point, hydrogen partial pressure in both arabinose and glucose assays was roughly $1.2x10^4$Pa (@70ºC), which corresponds to a dissolved hydrogen concentration of 105 µM (@70ºC). After this point, hydrogen production was not significant even though only 35% of arabinose and 13% of glucose were not utilized at the end of the experiment. The same final hydrogen yield, i.e. 0.7 molH_2 per mole of substrate was obtained

for NHR arabinose and glucose experiments (Table 7.2). Hydrogen production from arabinose could be increased in assays in which hydrogen partial pressure in the headspace was kept low.

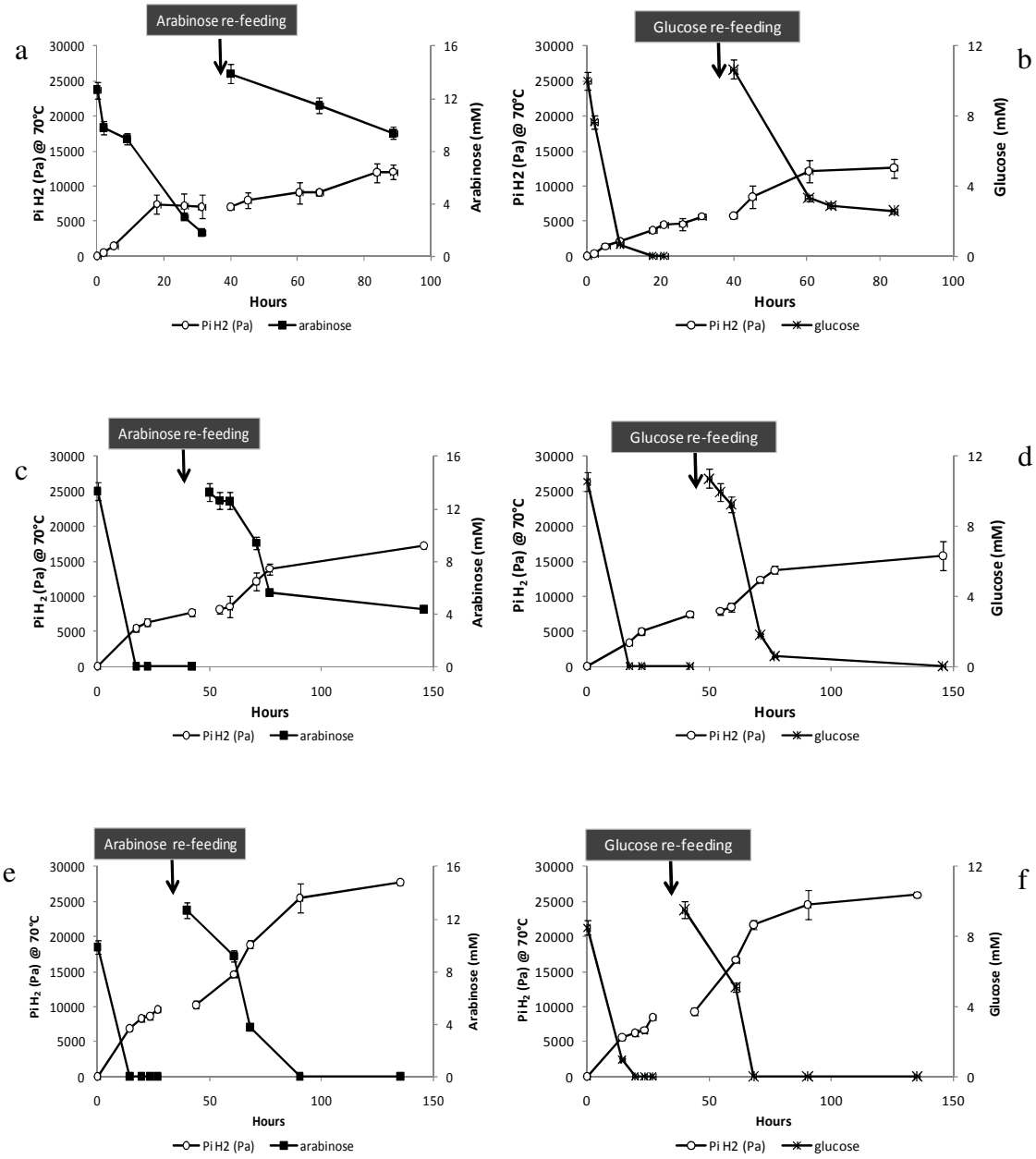


Figure 7.4. - Time course of hydrogen production and substrate consumption. a), b) batch experiments at pH 5.5 without head-space replacement. c), d) batch experiments at pH 5.5 with head-space replacement. e), f) batch experiments at pH 7 with head-space replacement.

A cumulative hydrogen production of 1.6-1.7x10^4Pa (@70ºC) was attained in HR arabinose experiments at pH 5.5 (Figure 7.4 c). This value is significantly higher than the one obtained in NHR experiments (p<0.0036: t-test), and corresponds to an increase of about 40% in hydrogen production. However, the highest hydrogen cumulative production was observed in HR arabinose assays performed at pH 7 (cumulative hydrogen production of 2.8x10^4 Pa @70ºC (Figure 7.4 f). This corresponds to an increase of 62% of hydrogen production compared to arabinose HR assays at pH 5.5. Arabinose was totally consumed in HR assays at pH 7, while in HR assays at pH 5.5 approximately 16% of the arabinose was not used (Figure 7.4 c, e). Nevertheless, non-consumed arabinose in HR at pH 5.5 was considerably lower than in NHR assays (Figure 7.4 a, c).

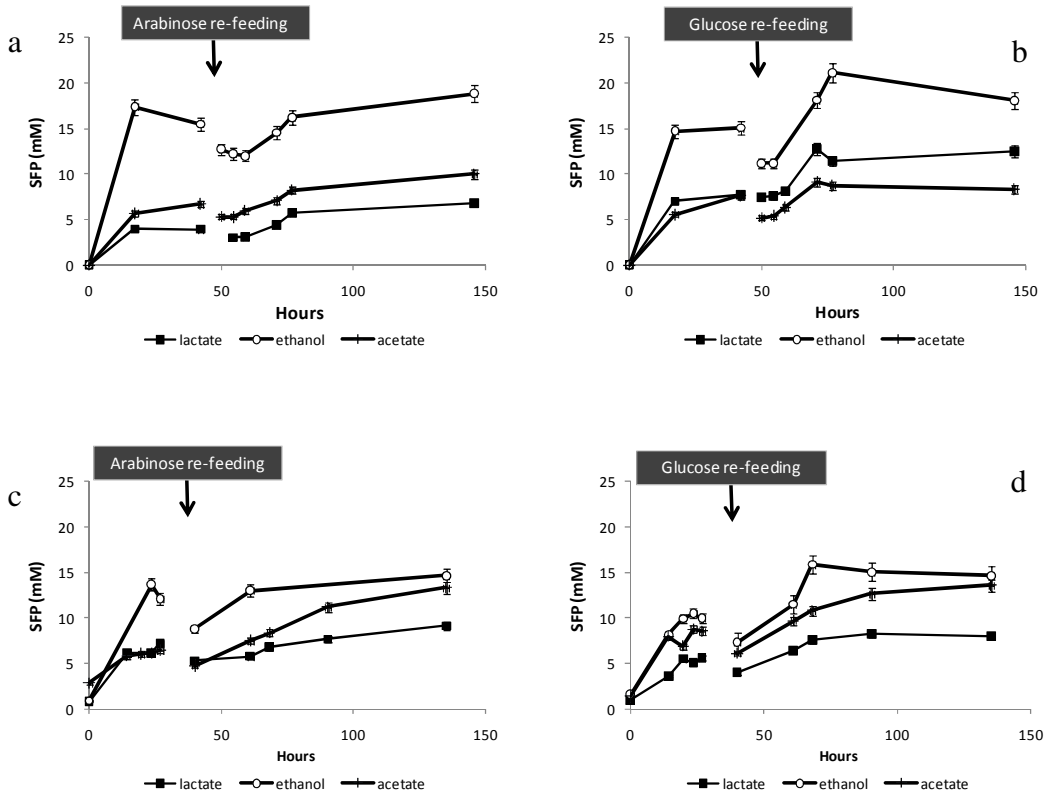


Figure 7.5. - Time course of soluble fermentation products. a), b) batch experiments at pH 5.5 with head-space replacement. c), d) batch experiments at pH 7 with head-space replacement.

Table 7.1. - Process performance of $R_{arab,}$ R_{gluc} and $R_{gluc+arab}$

	Feed concentration (mM)	Glucose utilization (%)	Arabinose utilization (%)	Hydrogen yield ($molH_2$ mol substrate consumed $^{-1}$)	Reference
Glucose Reactor (R_{gluc})	8.3	100	na	0.34±0.05	This study
	13.8	100	na	0.80±0.03	
	27.7	100	na	0.75±0.07	
Arabinose Reactor (R_{arab})	10.0	na	100	0.23±0.01	This study
	16.6	na	99	0.77±0.02	
	33.3	na	79	1.10±0.01	
Glucose + Arabinose Reactor ($R_{gluc+arab}$)	13.8+16.6	100	75	0.77±0.05	(Abreu et al., 2010)

Hydrogen yields in HR arabinose experiments at pH 5.5 and pH 7 were 0.76 and 1.15 mol H_2 per mole of substrate consumed, respectively (Table 7.2).For the HR experiments with glucose, the increase in hydrogen production was not significant when compared to NHR experiments. However, the HR experiments at pH 7 using glucose as substrate showed significantly higher (p<0.0078: t-test) cumulative hydrogen production compared to HR at pH 5.5 (Figure 7.4 d, f). Hydrogen cumulative pressure in HR glucose assays at pH 7 was of $2.6x10^4$ Pa @70ºC (Figure 7.4 f). Glucose was totally consumed in HR assays both at pH 5.5 and pH 7. Hydrogen yields in HR arabinose experiments at pH 5.5 and pH 7 were 0.6 and 1.4 mol H_2 per mole of substrate consumed, respectively (Table 7.2).

Table 7.2. - Substrate consumption and hydrogen yields from batch experiments

		Non Head Space Replacement (NHR)	
pH	Substrate	Substrate consumed (%)	Yield ($molH_2$ mol of substrate $consumed^{-1}$)
5.5	arabinose	65	0.68±0.05
	glucose	87	0.67±0.13

		Head Space Replacement (HR)	
pH	Substrate	Substrate consumed (%)	Yield ($molH_2$ mol of substrate $consumed^{-1}$)
5.5	arabinose	84	0.76±0.06
	glucose	100	0.58±0.07
7	arabinose	100	1.15±0.03
	glucose	100	1.36±0.14

For both pHs tested the main by-products formed were acetate, lactate and ethanol (Figure 7.5). At pH 5.5 approximately 20 mM of ethanol was produced from both substrates. At pH 7, ethanol formation did not exceed 15 mM. Acetate formation from both substrates at pH 7 achieved approximately 14 mM. In the case of glucose a decrease in 40% of lactate formation was also observed in incubations at pH 7.

7.4 Discussion

Continuous hydrogen production in EGSB reactors

R_{arab} and R_{gluc} showed a very similar performance during periods I and II of operation. However, when a higher organic loading rate was applied to the reactors (period III of operation, arabinose and glucose concentrations of 33.3 and 27.7, respectively), R_{arab} showed a steady state hydrogen production rate 1.6x higher than R_{gluc}. Furthermore, hydrogen production rate measured in R_{arab} was 1.3x higher than the one reported by Abreu et al. (2010) when feeding a EGSB reactor with a mixture of arabinose and glucose (1/1). Hydrogen production yield in R_{arab} was 1.10 molH_2 per mole of arabinose consumed. This is a high hydrogen production yield when compared to yields obtained in R_{gluc} (0.75 molH_2 per mole of glucose) and $R_{gluc+arab}$ (0.77 mol H_2 per mole glucose+arabionse) (Table 7.1). According to these results the presence of glucose may possibly decrease the overall hydrogen yield in continuous operation, particularly when higher organic loading rates are applied. Lower hydrogen production observed in R_{gluc} is likely associated with high lactate production; during period III of operation, increment in glucose concentration to 27.7 mM caused an increase in lactate production up to 20 mM (Figure 7.2). Sugar catabolism via the Embden-Meyerhof pathway yields pyruvate and reducing equivalents in the form of NADH. Subsequently, pyruvate can be reduced to lactate with regeneration of NADH (in this route there is no formation of hydrogen), or it can proceed to acetyl-CoA with the production of reduced ferredoxin (Figure 2.2). During the regeneration of the reduced ferredoxin and NADH hydrogen is produced. However, recovering redox potential by pyruvate reduction to lactate (Table 7.3, reaction (1)) seems to be energetically more favorable than by its conversion to acetyl-CoA (Table 7.3, reactions (2) and (3)), especially at higher hydrogen partial pressures (Verhaart et al., 2010).

Table 7.3. - Gibbs free energy changes for some of the glucose and arabinose oxidation reactions. Standard Gibbs energies of formation of arabinose (in aqueous solution, pH 7 and 25ºC) were estimated from the structures of the compounds, using a group contribution method described by (Mavrovouniotis, 1991); standard Gibbs energies of formation of other compounds involved in the reactions were obtained from (Thauer et al., 1977).

Equation		$\Delta G^{0,}$ [a] (kJ reaction^{-1})	$\Delta G'$ [b] (kJ reaction^{-1})
Fermentative reactions			
$NADH + H^+ + pyruvate^- \rightarrow NAD^+ + lactate^-$	(1)	-25	
$NADH + H^+ \rightarrow NAD^+ + H_2$	(2)	+18	-39
$2\ ferredoxin(red) + 2\ H^+ \rightarrow 2\ ferredoxin(ox) + H_2$	(3)	+3	-25
Glucose oxidation reactions			
$1\ glucose + 2\ H_2O \rightarrow 2\ acetate + 2\ CO_2 + 2\ H^+ + 4\ H_2$	(4)	-216	
$1\ glucose \rightarrow 1\ butyrate + 2\ CO_2 + 2\ H^+ + 2\ H_2$	(5)	-264	
$1\ glucose \rightarrow 2\ lactate + 2\ H^+$	(6)	-197	
$1\ glucose \rightarrow 2\ ethanol + 2\ CO_2 + 2\ H^+$	(7)	-315	
Arabinose oxidation reactions			
$1\ arabinose + 1.67\ H_2O \rightarrow 1.67\ acetate + 1.67\ CO_2 + 1.67\ H^+ + 3.33\ H_2$	(9)	-192	
$1\ arabinose \rightarrow 0.83\ butyrate + 1.66\ CO_2 + 0.83\ H^+ + 1.66\ H_2$	(10)	-228	
$1\ arabinose \rightarrow 1.66\ lactate + 1.66\ H^+$	(11)	-172	
$1\ arabinose \rightarrow 1.66\ ethanol + 1.66\ CO_2 + 1.66 H^+$	(12)	-269	

[a] Gibbs free energies (at 25ºC) calculated at standard conditions (solute concentrations of 1 M and gas partial pressure of 10^5 Pa).
[b] Gibbs free energies (at 25ºC) calculated at standard conditions (solute concentrations of 1 M and gas partial pressure of 1 Pa).

The fact that microbial communities composition in the reactors did not change along the three operational periods (Figure 7.3), suggests that the higher concentration of lactate produced in R_{gluc} during period III was related to changes

in metabolic pathways used by the microorganisms and not a consequence of bacterial community shift.

Two of the predominant DGGE bands present in the reactors sludge could be affiliated with *Thermoanaaerobacterim thermosacharolyticum* (similarity higher than 99%). A draft genome of *T. thermosacharolyticum* (Joint Genome Institute) allowed a search of genes that encode metabolic enzymes involved in pyruvate conversion. A L-lactate dehydrogenase (EC 1.1.1.27) is present indicating the possibility of pyruvate reduction to lactate. Some genes codifying subunits of enzymes related to pyruvate-ferredoxin oxidoreductases and NADH oxidoreductases are also present but a complete picture on the mechanisms involved in pyruvate conversion to acetyl-CoA cannot be retrieved. Clones corresponding to other predominant DGGE bands present in reactors sludge, exhibited highest sequence identity with *Klebsiella* sp. (99%), *Bacillus coagullans* (99%) and *Sporolactobacillus nakayamae* (95%). All these microorganisms are able to produce hydrogen and lactate, among other products, from a variety of carbon sources (Altaras et al., 2001; Kotay and Das, 2007; Wu et al., 2008). No genomic information is available for these species. Besides that, physiological information is sometimes contradictory. For instance, the presence of *Bacillus coagullans* in hydrogen producing reactors has been associated to the increase of lactate production (Karadag and Puhakka, 2010a; Karadag and Puhakka, 2010b), but other authors associated their presence to optimized hydrogen production (Kotay and Das, 2007; Kotay and Das, 2010).

The main possible reactions for the fermentation of arabinose and glucose and the calculated Gibbs free energy of global reactions are shown in the Table 7.3 (equations (4) to (13)) (only reactions yielding experimentally detected SFP in R_{gluc} and R_{arab} are represented). From a thermodynamic point of view lactate formation from glucose and arabinose is less favorable when compared, for example, to butyrate or ethanol production. However, in continuous processes lactate was one of the main SFP present in both reactors, especially in R_{gluc} at higher feed concentration (27.7 mM), and this might be related to the need of recycling reducing power from NADH as previously explained. The higher lactate

formation observed in R_{gluc} compared to R_{arab} during the application of higher loading rate could be associated to the higher NADH formation. Since it was verified in continuous (Figure 7.1 Figure 7.2) and also in batch experiments (especially after the second substrate addition) that glucose was metabolized faster than arabinose (Figure 7.4), this probably contributes to the accumulation of reductants inside the cell. It was observed that 5 days after the increase of substrate concentration, the R_{arab} had consumed 22.69 mM of arabinose, assuming the combination of pentose phosphate and Embden-Meyerhof as main pathway for the pentose metabolism to pyruvate, it was produced 36.65 mM of NADH. After 3 days of the substrate increment R_{gluc} had consumed 21.14 mM of glucose and, assuming that Embden-Meyerhof was the main pathway, 42.28 mM NADH were produced. It has been proposed that thermophiles usually possess some escape routes to dispose of reductants to prevent obstructions in their metabolic flux. One such route is the production of more reduced organic compounds like lactate (Kotay and Das, 2007; Verhaart et al., 2010). The lactic acid pathway appears to operate as a less efficient alternative to allow the oxidation of NADH. Li et al., (2010) suggest that the hydrogen production of *Thermoanaerobacterium* sp. falls into the oxidative decarboxylation pathway of pyruvate to acetylCoA that is NAD(P)H-dependent. The NADH pathway evolves hydrogen by hydrogenase activity through re-oxidation of NADH that is produced from glycolysis. When the concentration of hydrogen increases, hydrogen is primarily generated from reduced ferredoxin, since generation from NAD(P)H becomes less favourable. A switch to lactate formation is observed as a mechanism of reductant disposal and NAD(P)H oxidation (Shaw et al., 2009; Verhaart et al., 2010).

Hydrogen partial pressure and pH influence on hydrogen production yields

Hydrogen is known to restrict growth of thermophiles and it inhibits its own production at the level of hydrogenase (Rogers, 1986). In this study, the effect of hydrogen partial pressure was tested on the hydrogen production from arabinose and glucose, separately. It was verified an increase of hydrogen

production from both substrates tested when hydrogen was not allowed to accumulate in the head-space. The increase of hydrogen production observed in case of glucose, was not associated to the increase of hydrogen production yield, but to a higher substrate conversion. This suggests that the increase of hydrogen production was only a consequence of the enhancement of sugar utilization and not a result of more efficient yield. Even when hydrogen was not allowed to accumulate in the head-space, arabinose was not completely consumed. This could indicated that other limiting factors than PH_2, such as liquid by-products inhibition, were involved in hydrogen production from arabinose.

The effect of hydrogen partial pressure in several pure cultures of thermophiles was already described (Rogers, 1986) but the effect in mixed cultures is not completely clear. It has been reported that thermophilic hydrogen producing microorganisms could be inhibited by very low hydrogen partial pressure (0.1 $x10^4$Pa to 7.5$x10^4$Pa) (Kengen et al., 1996). Values of hydrogen partial pressure of $2x10^3$ Pa, $1.6x10^3$ Pa and $1.0x10^4$ Pa were described as inhibitory for hydrogen production with *Thermotoga maritime*, *Pyrococcus furiosus* and *Caldicellulosiruptor saccharolyticus*, respectively (Adams, 1990). In the present study using glucose and arabinose as substrates and an extreme thermophile mixed culture, it was observed an inhibitory effect of PH_2 similar to C. *saccharolyticus* ($1.2x10^4$ Pa@70ºC).

The effect of using higher pH than what was used in continuous operation (pH 5.5) was also evaluated. pH of 7 (6.5@70^oC) was tested in batch for hydrogen production from arabinose and glucose. Higher cumulative hydrogen production and yields were obtained from both substrates using pH7. Comparing to pH 5.5, it was verified a decrease in ethanol production and increase in acetate from both substrates. Lactate formation from glucose was also less than what was observed at pH5.5. In this study, pH 7 revealed to be more efficient in terms of hydrogen production and yields than pH 5.5, for both substrates (glucose and arabinose). The lower hydrogen production observed at pH 5.5 was associated to high ethanol and low acetate production (Figure 7.5).

The influence of pH has been recognized as a key factor in determining the outcome of hydrogen fermentation. It has been referred that higher pH avoids the shift to solventogenesis, consequently decreasing hydrogen production associated to this process. It has been suggested that the shift to solvent production in some *Clostridium* sp. is an adaptive response of the cell to inhibitory effects produced by acid end products (van Niel et al., 2003). Lower pH resulted in higher concentration of acids under undissociated form resulting in a rapid induction of solventogenesis witch was accompanied by the decrease in the rate of hydrogen production (Valdez-Vazquez et al., 2006). The present study suggested that the maintenance of pH close to neutral (6.5@70^{o}C) could be an extra aid to avoid hydrogen lost by the production of more reduced organic compounds at extreme-thermophilic conditions.

More than the hydrogen partial pressure, in the present study, the pH was a very important variable determining an efficient hydrogen production from arabinose and glucose. The results obtained in batch provide useful indications for optimizing hydrogen production in continuous process, though considering the differences between the two operating modes. In fact, different SFP composition were obtained in the batch experiments and in the continuous reactors. The batch experiments permits the accumulation of SFP which could lead to different conditions and subsequently induce different metabolic pathways (Schafer and Schonheit, 1991; Schroder et al., 1994; van Niel et al., 2003).

7.5 Conclusions

In continuous mode, hydrogen production rate was lower when glucose was used as substrate at higher loading rates, compared to arabinose. This fact was associated to the higher lactate production. The higher concentration of lactate was not a consequence of bacterial community shift, but a change in the main metabolic pathways of glucose catabolism.

The batch experiments have shown that the effect of hydrogen partial pressure on hydrogen production from glucose was related to sugar utilization and not to

hydrogen production yield. Furthermore, the utilization of pH 7 revealed more efficient in terms of sugars uptake, hydrogen production and yield than pH 5.5, with both arabinose and glucose.

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General conclusions and future work

Chapter 8

8.1 General conclusions and final remarks

In this thesis, different strategies and operating conditions for an optimal fermentative biohydrogen production were studied.

Biological hydrogen production is affected by several environmental factors such as pH and substrate concentration. Therefore, batch assays were performed to compare the effect of these parameters on the biohydrogen production from arabinose using different types of inoculum. Arabinose was used as substrate, since this sugar is one of the components of hemicellulosic residues and is referred as having low hydrogen production efficiency due to its complex fermentation pathways. Higher hydrogen production rates and yields were attained for arabinose concentrations lower than 40 g L^{-1} and initial pH values between 6 to 8, either for suspended or granular biomass. Granular sludge was less affected by increasing arabinose concentration than suspended sludge, probably due to the existence of a concentration gradient within the granule that might contribute to the exposure of the hydrogen producing populations, located just beneath the surface, to lower arabinose or metabolic byproducts concentrations. Moreover, smaller lag phases preceded the onset of hydrogen production and arabinose was consumed more extensively in the assays inoculated with granular biomass. Hence, granular sludge appears to be the best inoculum for continuous biohydrogen production processes.

Microbial selection and development of H_2 producing granules from suspended biomass, requires a long start-up time. Alternatively, selecting the microbial communities present in matured/developed anaerobic granules towards hydrogen production is a potential strategy for faster start-up of high-rate H_2 producing reactors as it eliminates the need for a granulation period.

Heat and chemical treatment of previously formed methanogenic granules to suppress H_2-consuming microorganisms affected both micro- and macro scale structure and microbiology of granular sludge. Pretreatment and subsequent pulses with BES revealed to be the best strategy for high-rate reactors start-up and further stable continuous operation. This technique was effective in

extinguishing the methanogenic hydrogenotrophic activity and did not extensively affect macro- and microstructure of the granules. Homoacetogenic activity, however, could not be completely inhibited.

Significant improvement of hydrogen production and process stability was further obtained by using an "engineered inoculum", where known hydrogen producers were co-inoculated with heat-treated granular sludge in an EGSB reactor at extreme thermophilic conditions. The contact of heat treated granules with an enriched active hydrogen producing culture during the reactor start-up, contributed to the development of a stable and efficient hydrogen production during the engineered system operation. The presence of potential hydrogen producing bacteria clustering within the *Clostridium* and *Klebsiella* genera is likely related to the better performance exhibited by the engineered system. In the engineered system homoacetogenesis was not observed.

A key step on the biohydrogen production from plant biomass involves the presence of microorganisms capable of efficiently fermenting hexoses and pentoses. The combined fermentation of mixtures of hexoses and pentoses is often prevented due to catabolite repression; in the presence of glucose, pentoses might be converted to a lesser extent thereby decreasing overall fermentation yields, as it can be verified in chapter 6. Furthermore, efficient hydrogen production from sugars is dependent on the different possible fermentation pathways.

The conversion of a C5-sugar (arabinose) and a C6-sugar (glucose) to hydrogen, using anaerobic mixed-cultures under extreme thermophilic conditions (70ºC) was studied. Higher hydrogen production rate was obtained using arabinose at higher loading rates comparing to glucose. The lower hydrogen production observed using glucose at higher loading rates, was associated to higher lactate production. It was verified that the higher concentration of lactate was not a consequence of bacterial community shift, but a change in the main metabolic pathways of glucose catabolism. Additionally, in batch mode it was verified that the effect of hydrogen partial pressure on hydrogen production from glucose was related to sugar utilization and not to hydrogen production yield. Moreover,

the utilization of pH 7 revealed more efficient in terms of sugars uptake, hydrogen production and yield than pH 5.5, with both arabinose and glucose.

8.2 Suggestions for future work

The work described in this thesis adds significant insights into the optimization of biohydrogen dark fermentation process. Future work needs to aim at the inhibition of microorganisms and pathways that result in the consumption and/or no production of hydrogen. Optimization of reactors operational conditions and configuration as well as, the development of combined processes are of utmost importance.

One of the main hydrogen consuming processes, along with methanogenesis, is the homoacetogenesis. The occurrence of homoacetogenesis decreases drastically the overall hydrogen production. Therefore, a better understanding of this process as well as, on the microorganisms involved and inhibitory conditions is needed. The utilization of advanced molecular tools for the characterization and quantification of microbial consortia developing during homoacetogenesis as well as, the use of marked substrate, can give more insights into the biochemistry of this process.

More effort should be put into screening of extreme environments for efficient hydrogen-producing inocula. The ability of these unusual microorganisms to thrive under extremes of temperature, pressure, pH or salinity, give them a competitive advantage over other during enrichment procedures. Furthermore, extreme operation conditions can be applied in order to better suppress non-desired hydrogen consuming microorganisms without affecting hydrogen-producers, optimizing hydrogen production. This consideration should be further explored.

Optimization of operational conditions might be achieved by the utilization of modeling methods. The metabolic pathways and fluxes are relatively well understood for pure culture fermenting a defined substrate. This type of analysis

can be use for modeling fermentations of complex substrates by microbial consortia involving multiple metabolic types with unknown interactions.

Scientific output

The overall work presented in this PhD thesis gave origin to the following publications:

PAPERS IN JOURNALS WITH PEER REVIEW:

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